





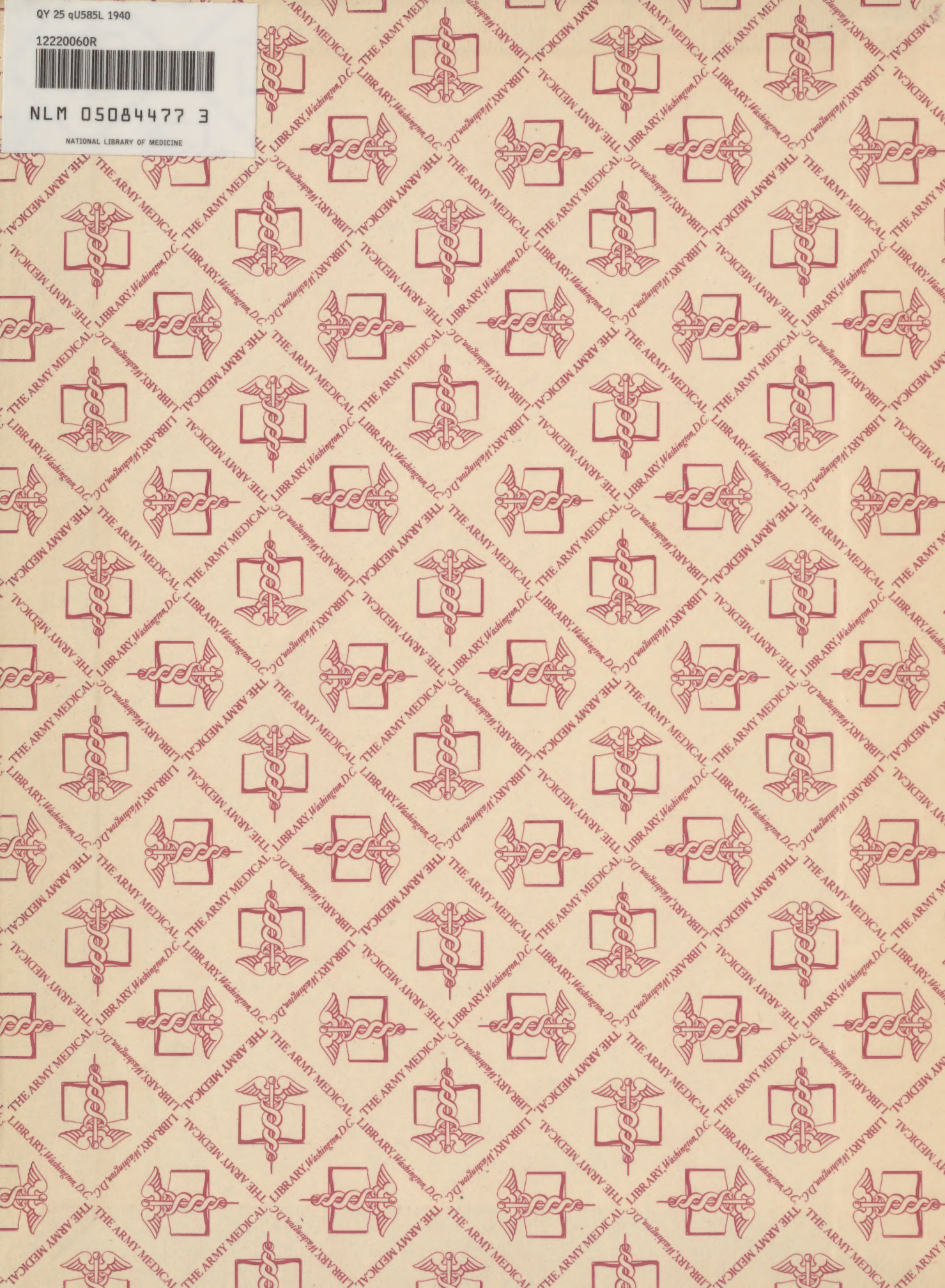
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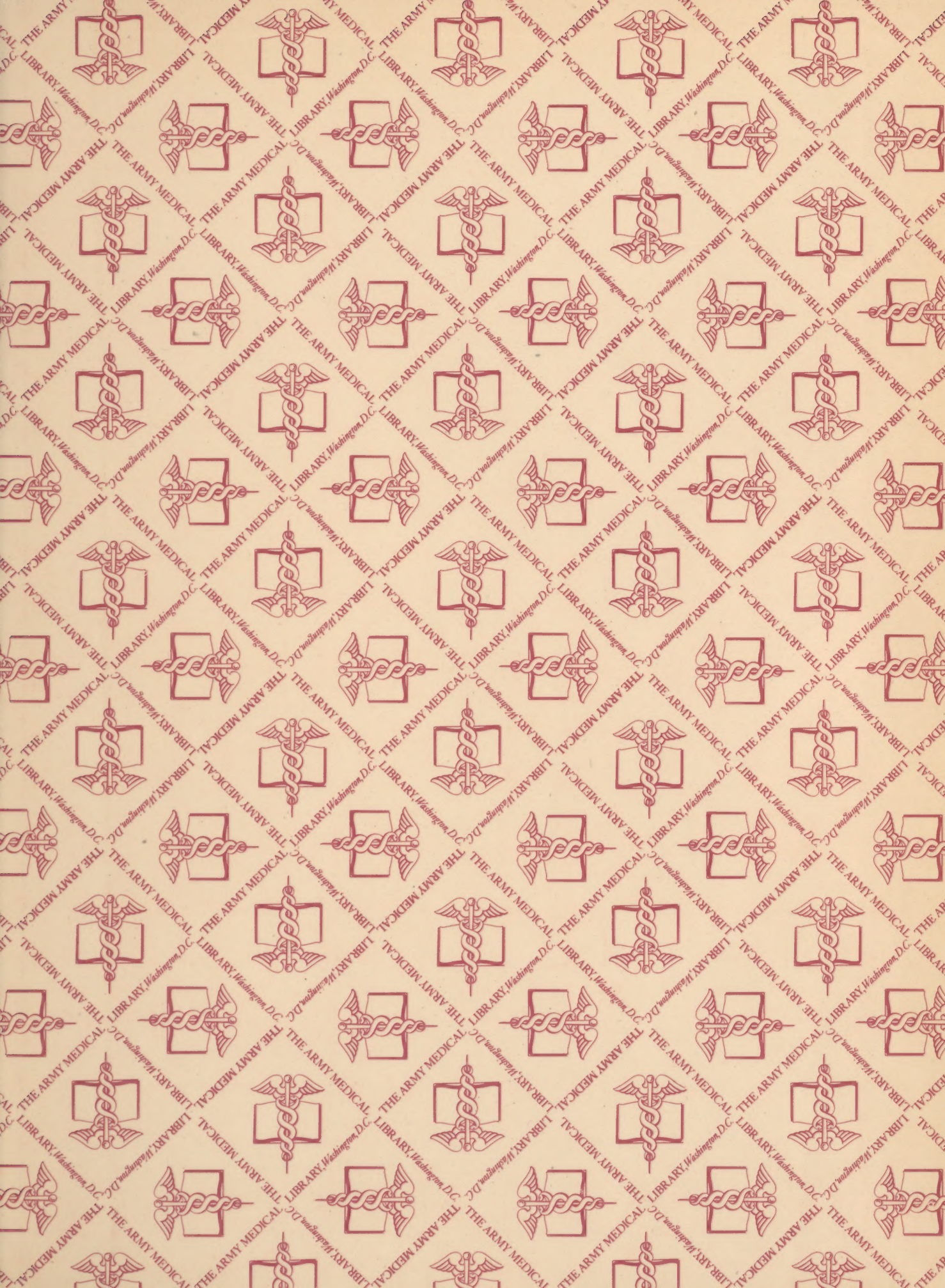


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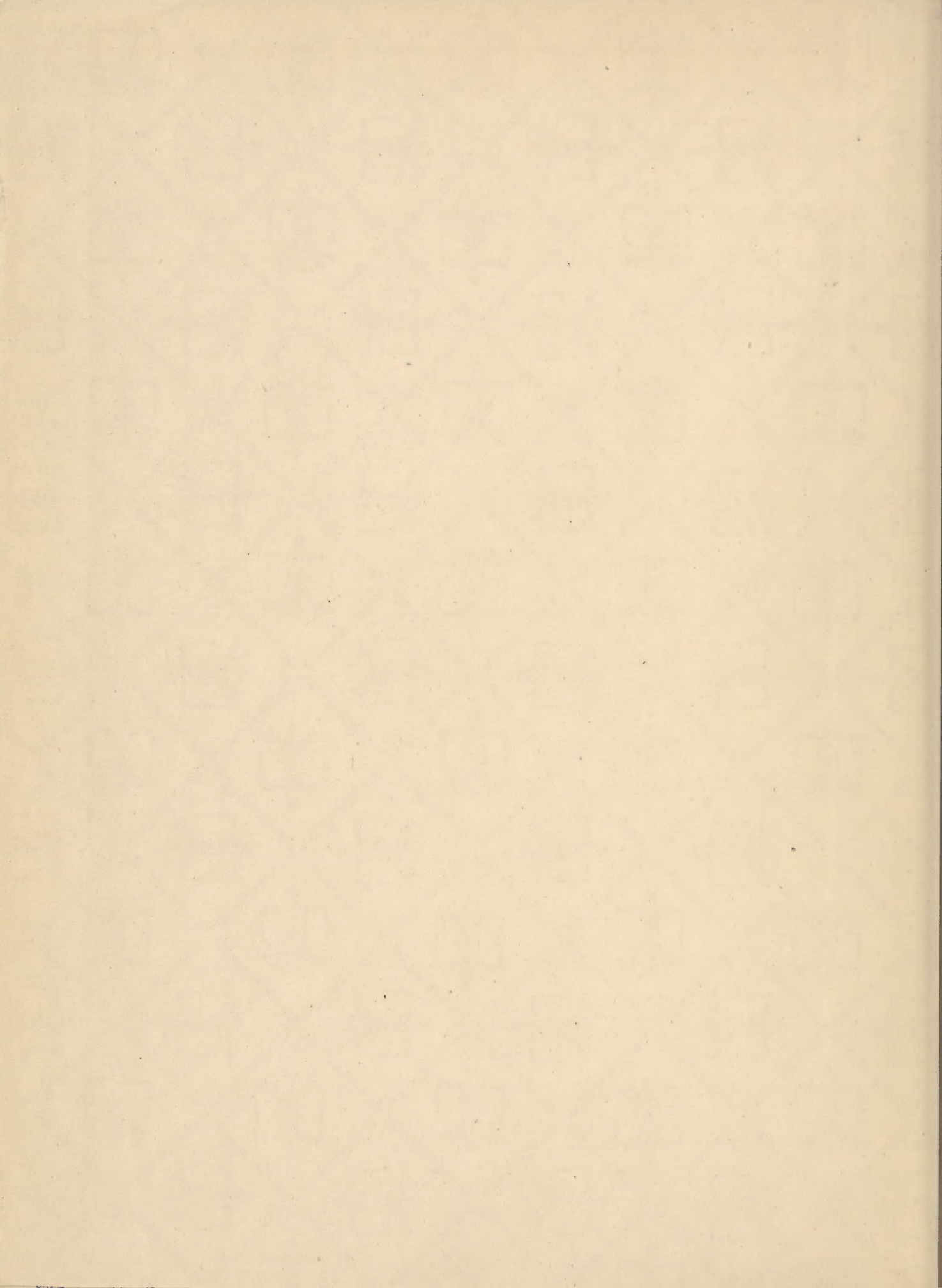
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# LABORATORY MANUAL



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## The Compound Microscope

Structure: A microscope, the working tool of a microbiologist, consists of four groups of parts, each group composed of a number of units:

### 1. Framework:

- a. Base, on which the microscope rests.
- b. Handle, by which it is carried and which supports the magnifying and adjusting systems.
- c. Stage, a perforated horizontal shelf on which the object rests.
- d. Mechanical stage, which moves the object about on the stage.

### 2. Illumination system:

- a. Mirror, which reflects light upward.
- b. Condensor, placed just beneath hole in stage.
- c. Diaphragm, just beneath condensor, controlled by a small button to open or close it, in controlling the light intensity.

### 3. Magnification system, through which the light passes:

- a. Nosepiece, generally triple, to receive the objectives.
- b. Objectives, generally three, the main magnifying part, designated according to their focal distance as 16, 4 and 1.9 millimeters, the latter being the highest power and used in most bacterial studies.
- c. Body tube, and Drawtube, through which the light passes to ocular.
- d. Ocular, an additional magnifying piece, of which two are generally furnished, 6.4X giving somewhat less magnification than 10X (number indicates times object is magnified).

### 4. Adjustment system which moves body tube up or down for the focusing of the objective to the object.

- a. Coarse adjustor gives rapid movement over a wide range and is used to obtain an approximate focus.
- b. Fine adjustor gives a very slow movement over a limited range and is used to obtain an exact focus, after prior coarse adjustment.

The magnification of any combination of objectives and oculars may be obtained by multiplying the magnification of the objective by that of the ocular. The magnification of the objectives differs slightly with the quality of the lens and is stamped on each objective. (4mm. - from 43 to 45 X; 1.9mm. from 93 to 97X).

The magnification given by different combinations of many objectives and oculars is as follows:

	<u>6.4X</u>	<u>10X</u>
16 mm. (10X)	X 64	X 100
4 mm. (43X)	X 275	X 430
1.9 mm. (95X)	X 610	X 950



### Use of the Microscope:

1. Adjustment of the light: A suitable light source, intense for the higher magnification, is placed in front of the microscope; this may be daylight (not sunlight) or a bright artificial light. The mirror is adjusted to direct this light upward through the condensor. Having attained a bright light through condensor, this light may be reduced to the desired intensity by closing the diaphragm.
2. Adjustment of the object: The material to be examined, on a glass slide, is placed on the stage, held in the grip of the mechanical stage and moved around by it until the desired areas lie beneath the objective.
3. Adjustment of the magnification system: The desired objective is rotated into place at lower end of the body tube. The desired ocular is placed in the upper end of the drawtube. The observer then closely applies an eye to the ocular.
4. Adjustment of focus: With the coarse adjustment screw, the tube is placed at the proper approximate position. Each objective requires a distance in millimeters between the object and the lower part of the objective, corresponding to the number of that objective (which is its focal distance): the 1.9 objective is placed at about 1.9 millimeters ( $1/12$  inch) above the object. This objective (1.9), but not the 16 and 4, requires that there be a drop of cedarwood oil between object and objective. Having gained this approximate focus, the observer's eye applied to the ocular further guides the coarse adjustment to a more approximate focus, until the microscopic object can be roughly seen. The fine adjustment is then used to give an exact focus, providing a clear image. A readjustment of the light intensity may then be made to give the maximum visibility. In general it is best to focus upward, for the beginner who focuses downward may force the objective into the object with great force and break it.

### Care of the Microscope:

1. Objective and ocular surfaces may be cleaned by a little breath moisture, followed by stroke of lens paper, as in cleaning spectacle glass.
2. Cedarwood oil, used on objective, is to be wiped off after each use, with soft lens paper, avoiding gauze or other scratching agents.
3. Cleaning of entire microscope is to be done frequently, to remove dust, finger marks, oil, grease or specimen remnants.
4. Cover is to be provided at all times when microscope is not in use.
5. Dried oil may be removed by wiping with lens paper soaked with xylol, wiping away surplus at once with dry lens paper. Caution must be used in applying any solvent fluid to the objective; alcohol should never be used.
6. Housing of the ocular tubes should never be removed, except by expert, for slight maladjustment of the contained prisms will distort images.
7. Light machine oil is applied to working parts occasionally.



## PREPARATION OF GLASSWARE

Cleaning: 1. New Glassware: Boil in water to which has been added sufficient white soap or washing soda (type supplied for dish washing machines) to provide a good foam. Cool water to 45° to 50°C. and wash thoroughly inside and outside, using a wash rag and the proper type brush. Rinse thoroughly in running tap water, preferably hot. Re-rinse in distilled water and invert on drain board or place in hot air oven at 160°C. to dry.

2. Used Glassware: Sterilize test tubes, flasks, etc., that have contained cultures of pathogenic bacteria, in autoclave, or by placing in a 3 to 5% solution of cresol for several hours, and empty contents into sink (broth cultures) or garbage can (agar cultures). Glassware smeared with petrolatum, paraffin or wax pencil should be given a preliminary cleansing with xylol. Wash as described for new glassware but use greater care to cleanse inside of articles.

3. Dichromate-sulphuric acid cleaning solution: Cloudy glassware, which cannot be cleansed by soap and water, and used pipettes and slides, after preliminary washing, should be soaked in this solution in a large glass or earthenware container for two to twenty-four hours. Formula;

- a. Sodium dichromate (bichromate), technical ----- 120 gms.
- b. Tap water ----- 1500 cc.
- c. Dissolve a in b, filter and add,
- d. Sulphuric acid (commercial) ----- 180 cc.

4. Cleaning Special Glassware: a. Pipettes: Immediately after use, place in tall jars containing 2 to 5% cresol solution. To wash inside of pipette and force out cotton plug, attach rubber tube to cold water faucet and insert the tip-end of pipette into the hose; turn on water and force it through pipette until clean; rinse in distilled water and dry. Inspect pipettes, pick out any that are not perfectly clean and soak overnight in above acid cleaning solution.

b. Used Glass Slides and Cover Slips: After use, place in jar of 5% cresol solution, as a cleansing disinfectant. Wash in hot running water; rub individual slides, between thumb and forefinger with soap scouring powder and rinse in tap water. Either soak overnight in acid cleaning solution described above, rinse in tap water, followed by distilled water and alcohol or rinse in distilled water and soak in alcohol containing 2% acetic acid; dry and polish with soft cloth.

c. Syringes: Wash in tap water immediately after use for withdrawing blood, or fill with cresol solution for several hours after contamination with pathogenic bacteria. Wash, rinse and dry.



Plugging, Wrapping and Storage:

1. Pipettes: Plug base of each loosely with cotton. Wrap individually with paper and sterilize with dry heat.

2. Syringes: Dismantle syringe and wrap in heavy unbleached muslin. Smaller syringes may be sterilized in large cotton-plugged test tubes.

3. Test tubes: Plug with cotton.

4. Flasks: Plug with cotton wrapped in cheesecloth.

5. Centrifuge tubes: Plug firmly with cotton contained in piece of cheesecloth large enough to leave apron about one inch wide around mouth of tube.

6. Petri dishes: Are placed in round or square metal boxes for sterilization, or may be wrapped in paper.

7. Sterilize, label and store in cabinet protected from dust.

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Sterilization may be secured by dry heat, steam under pressure, steam not under pressure, boiling in water, chemicals, or by filtration.

1. Dry Heat: A temperature of 160 to 190°C. in a gas or electric oven for at least one hour is sufficient for the sterilization of small articles. A prolonged exposure to temperature over 175°C. will char or burn cotton and paper. The following rules apply for routine use:

- a. Sterilize glassware, wrapped in cloth or paper or plugged with cotton, by heating for minimum of two hours at 160° to 175°C.
- b. Sterilize glassware, closed with glass stopper or packed in metal containers, by heating for minimum of two hours at 160 to 190°C.
- c. Glassware, closely packed or in large container, must be heated for longer period of time to ensure penetration of heat to and sterilization of central portion.

2. Steam under pressure (autoclave): The standard laboratory autoclave is an unjacketed, horizontal type, set at 15 to 17 lbs. pressure. It is used for the sterilization of linen, cotton goods, rubber, glassware and culture media which are not injured by high temperature, and for killing old cultures. Routinely, sterilize for 15 minutes at 15 lbs. pressure. Large packages or media in bulk will require from 30 minutes to one hour; example: 600 cc. of medium in 1000 cc. flask requires 30 minutes, the additional time attaining penetration of the bulky material to the sterilizing temperature;

- a. Place material in autoclave, leave door open, open escape valve and turn on steam.
- b. Close door when steam starts to flow from autoclave;
- c. Leave escape valve open until steam escapes rapidly, then close, leaving crack wide enough for trickle of steam to escape.
- d. Allow pressure to rise to 15 lbs. and sterilize for desired length of time.

3. Steam not under pressure: The Arnold sterilizer is used for materials, such as nutrient gelatin, milk media and media containing carbohydrates, that may be damaged by overheating,

- a. Place sterilizer over good gas burner and bring water in pan to a boil.
- b. Remove top, or open door, and place media in inner compartment. Close,
- c. Heat for 20 to 30 minutes,
- d. Leave media at room temperature and repeat the procedure on the two succeeding days.

4. Boiling in water: Syringes, used for injections or drawing blood, and dissecting instruments are sometimes sterilized by boiling in water for 30 minutes,

5. Chemicals:

- a. A 2 to 5% solution of compound cresol may be used to sterilize discarded cultures, used glassware, or rubber gloves and for disinfecting laboratory floors and table tops.



b. Bacterial antigens and vaccines may be sterilized with 0.2% formalin or 0.5% phenol.

c. Antisera may be sterilized by addition of 0.5% phenol and normal serum for preparation of Loeffler's medium by addition of 2% chloroform.

6. Filtration: Berkefeld, Mandler, Seitz or other filters are employed for removing bacteria from liquids that may be damaged or destroyed by heat, such as antisera, dextrose solution and ascitic fluid. Process described in section on viruses.

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## FEEDING, CARE AND BREEDING OF

### LABORATORY ANIMALS.

Laboratory Animals: (1) rabbit, (2) Guinea pig, (3) mouse, (4) albino rat and (5) monkey.

Reception Quarantine: All animals received from an outside source, should be isolated for 10 days to 3 weeks in previously disinfected quarters, and found to be free from disease before mixing with regular stock.

Housing: Animal quarters should be kept clean, dry and completely free from vermin. The optimum temperature for most animals is 65° to 70 F. with adequate ventilation. The standard large (10½") and small (8") animal jars are suitable for mice and rats; the large jar also can be used for a small guinea pig. The standard galvanized iron animal cage (14" x 14" x 16") will hold one rabbit or several guinea pigs. For use in breeding rabbits or guinea pigs, larger cages or pens, preferably with outside runways, should be built. The bottom of the jar or tray in cage should contain an absorbent bed material, such as wood shavings; hay or straw may be used in large breeding cages. Clean quarters and renew bedding twice per week.

#### Rabbits:

(1) The diet recommended consists of commercial "Rabbit Pellets" supplemented once or twice per week with feeding of green stuff, such as carrots, lettuce or celery tops. A diet consisting of equal parts of oats, wheat and barley, plus 10% of legume, soybean or linseed meal is suitable. Alfalfa or timothy hay will serve both for food and bedding. Always keep plenty of water and a small piece of rock salt in the cage.

#### (2) Diseases:

a. "Coccidiosis", an intense and fatal enteritis, is the most serious disease. Observe new rabbits for this several days before adding to stock.

b. "Ear Mange" is caused by a mite; can be cured by local application of a parasiticide.

c. "Snuffles" is a cold-like disease caused by a filterable virus. Isolate infected rabbits until 3 weeks after recovery.

(3) Breeding: Keep one male (buck) for each 8 to 10 females (does). Females are ready for mating at age of 10 months and may be bred every 3 months thereafter (4 litters per year). Keep record of date bred; gestation period 31 days; 2 or 3 days before expected arrival of litter place small breeding box and ample supply of bedding in cage. Wean young after 8 weeks and separate sexes.

#### Guinea Pigs:

(1) Feeding: Same as for rabbits, except they must have supplementary feeding of green stuffs to supply Vitamin "C", at least twice per week.

#### (2) Diseases:

a. Salmonella infections, chiefly Salmonella typhimurium and S. enteritidis, are most dangerous of common diseases. Best method of control: - Kill all potentially infected animals, sterilize room and cages and obtain new stock.



b. Vitamin "C" deficiency is caused by lack of sufficient green stuffs in diet. Characterized by coarse hair and mangy appearance. It is transmissible to young through mother. Treatment: - improved diet.

c. Balantidium coli type of enteritis.

(3) Breeding: Use colony breeding with 4 or 5 females in cage with one male; duration of pregnancy - 63 days. Wean young and separate sexes when 4 or 5 weeks old.

Mice: Several different strains used, such as, white mice, Swiss mice (also white) and C 57 strain (black).

(1) Feeding: Commercial dog or fox chow checkers furnish an ample, balanced diet for growth and breeding; occasionally add piece of carrot or other greenstuff. Must have supply of fresh clean water in cage at all times. Mice will do well on simpler diets, such as, (a) the mixed grain diet listed above for rabbits, or (b) dry bread with water or skimmed milk, with addition of cod liver oil once per week.

(2) Diseases:

a. Salmonella infections (mouse typhoid), caused by same organisms as for guinea pigs, are common and very dangerous. To control: - destroy all infected stock, sterilize room and cages and obtain fresh stock.

(3) Breeding: Colony breeding, with 4 or 5 females to one male; gestation period 21 days; when well advanced pregnancy is observed, place female in individual jar. After 21 days, isolate young and return mother to breeding jar. Feed young same as adults, but addition of evaporated milk to diet hastens growth.

Albino Rats:

(1) Feeding: Same as for mice.

(2) Diseases: If cages are kept clean and ample diet provided, rats are very resistant to disease.

(3) Breeding: Young females are ready for breeding when 4 months old. Use colony method of breeding with 4 females and one male in cage; duration of pregnancy, 22 days; not necessary to remove pregnant female from breeding cage. Wean young and separate sexes when 21 days old.

Monkeys:

(1) Feeding: Monkeys will do very well on "dog-chow checkers" plus canned tomatoes, with occasional feeding of fruits and nuts (oranges, apples, bananas, peanuts, sunflower seeds, etc.).

(2) Diseases:

a. Pneumonia, usually fatal.

b. Miliary tuberculosis.

(3) Breeding: In captivity in small laboratories is not practical.

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### Glass Handling

1. Cutting of glass tubing and test tubes.
2. Bending of glass tubing.
3. Making capillary pipettes from glass tubing.
4. Preparing an ampule out of a test tube.
5. Blowing of bulbs in glass tubing or test tubes.
6. Sealing joints in glass tubing.

In the average laboratory there is some call for turning glass tubing or test tubes into a form for some special purpose, and the faculty for doing this may be readily acquired with practise.

Equipment: Only soft glass can be so handled with the ordinary laboratory equipment; the hard glasses, such as "Pyrex" having too high a melting point to be handled without special high temperature blowpipes. The standard 6, 8, and 10mm, thick wall tubing and the standard test tube will suffice for the average requirements. While some simple manipulation may be done on a bunsen burner, it is preferable to have a blast lamp, both to provide higher temperature and to have smaller controllable heat point. Most blast lamps are designed for use with artificial gas; difficulty may be encountered in using natural gas, of higher B.T.U., with these burners and where natural gas is used special modification of the burners may be required. Satisfactory gasoline blast burners are also on the market. A small triangular file provides the means of cutting the glass.



### Cutting of Glass Tubing

Place the glass tubing on a table, hold it firmly and nick it in one spot by firmly drawing across it the edge of a triangular file. (It is rarely necessary to extend this file nick around the tubing.) Then holding the tubing in both hands with both thumbs opposite the nick a quick snap will complete a clean cut break of the tubing. If the break is not clean cut it indicates the need of deeper nick or of a modified manipulation in effecting the snap, a technic to be acquired with practice. If one end of the tubing is too short to so handle, the snap may be effected by holding the long end rigidly in one hand and hitting the small end by the file held in the other hand. After the break any sharp points, from failure to attain a satisfactory clean break, may be filed down. The surface of the break is finally smoothed by melting it in a hot flame; at this time, by overmelting it, the bore at the top can be reduced to any desired size.

Of course the hot glass should not be laid down on the table top; an asbestos board, the tip of a wire basket or a metal ring stand may be so used.

### Cutting a Test Tube

Make the file nick as for glass tubing but make it deeper and preferably encircle the tube. A thin tube may be broken at this point by a bi-manual snap. Thick tubes require additional aids to complete the break: at one point make the file nick especially deep, then touch the tube firmly at this point with the red hot file tip—a fracture should then result; if the fracture is not complete it may be traced around the tube by keeping the red hot tip just ahead of the fracture line, on a cold test tube. The trim up is the same as for tubing.

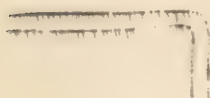
### Bending of Glass Tubing

Holding both ends place the tubing in a hot flame so that at least an inch gets hot. Rotate tube while it is heating to make the heat even on all sides. When the glass is red hot and soft remove from flame and bend to the desired form keeping it in that position until it has hardened. If a broad bend is desired, as in making a "U" bend, several inches should be so heated. If only a slight bend is to be made, an inch of glass will suffice. At first you will have a tendency to overheat the glass and draw the two ends apart, distorting the shape and caliber. Also if you underheat, or put forced pressure on the bending effort, an undesirable collapse of the tubing at the bend will occur. A satisfactory bend retains the same caliber throughout the tubing.



If, in working with thin tubing, the collapse at the bend cannot be prevented there is a device available for preventing it: having sealed one end of the tubing before melting it, the mouth is applied to the other end and while effecting the bend, and enough air pressure is made into the tubing to return the collapsed tubing to its proper form - a procedure similar to the blowing of glass bulbs described below.

#### Satisfactory Bend



#### Unsatisfactory Bend



#### Making Capillary Pipettes from Glass Tubing

Glass tubing is heated, rotated, in flame to softness, removed from flame and the two ends drawn apart and held in place until hardened. The size of the resultant capillary tubing will depend on the degree of heat, the rapidity of drawing out and the extent of the drawing out. The tendency is to make the tube too small by too rapid separation of a narrow length of heated tubing. There is frequent use in bacteriology for such a pipette in this form.



It is desirable to keep on hand 8 inch lengths of glass tubing, with cotton plugs at both ends, the whole sterilized by dry heat for use in making into pipettes as desired for special uses. Such pipettes may be given a narrowing of lumen about  $3/4$  inch from the end, to prevent the cotton plug from passing deeper into the pipette.

#### Preparing an ampoule out of a test tube

Proceed with a test tube just as above drawing, the two ends only about two inches apart resulting in a neck of about 4 mm. diameter. The tube may be cut at this point converting the lower end into an ampoule to be later sealed, or the test tube may be left intact to be later sealed at the constriction. Excessive heat may make tube difficult to hold; this may be avoided by placing a large perforated cork over each end and holding and rotating these.



### Blowing bulb in Glass Tubing

One end of the glass tubing is sealed and sufficient length allowed for holding one end in hand, the other end in mouth while blowing. Holding the tube over the flame with the hands rotating it for even heat, it is given a rod heat to the melting point, then passed to the mouth to blow up the bulb to the required size. If the bulb is of considerable size some concentration of glass must be attained before the final blow; this is done by gently approximating the two ends while the middle is soft, giving an occasional slight blow to prevent collapse of melted glass. Trouble encountered will consist of eccentric bulbs, due to uneven heating, or to thin paper shell bulbs due to overblowing without glass concentration. A test tube may be similarly handled. A terminal bulb may be made at end of glass tubing by a one hand manipulation and blowing.

### Union of Glass Tubing

If two straight pieces of glass are to be spliced, one piece is first given a seal at one end, the two pieces are given a slight flange or gently rotated within the flame, the seal end of the triangular file. The two flanges are made of same size, shape, and same grade of glass; they are heated to a red heat by the approximation to complete sealing on all sides, there results a union which is oversize and too thick; this union may be trimmed off to even size with the tubing by heating over the flame, while rotating evenly, blowing slightly into the tubing occasionally as tendency to collapse occurs, and slightly separating the two ends and blowing slightly if over thick glass is noted at the union.

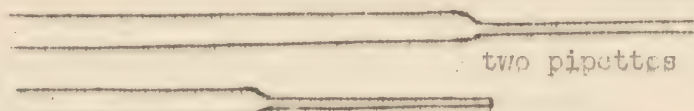
By similar procedures glass tubing of different size or glass tubing and test tubes may be united. A hard glass united with a soft glass would tend to fracture at the union. A union having thick spots or knobs will tend to fracture on change of temperature.



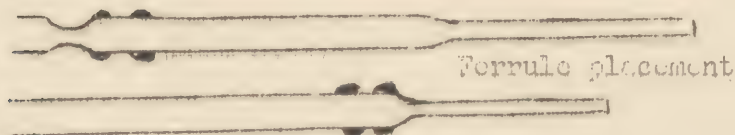
### Making joints in glass tubing

The making of Y of glass tubing, that is a three way tube, combines the process of blowing a bulb and union of two glass tubes. We begin with glass tubing of any desired size. Two lengths are prepared, each sealed at one end, such as by being drawn to capillary length. One of these is given a flange at open end, the flange molded into an oval shape by the action of end of file on the molten tube. The other piece of glass is given a hole in its center to receive the flange; the tubing is held in a small hot flame, without rotation, to heat only one small spot of the tubing; when it is melted a lopsided bulb is blown to an extreme thinness even to rupture; then the bulb is crushed away with the file leaving the hole surrounded by the ragged bulb fragments; heat is applied to the edges of this hole to make it evenly heated and the same size as the flange on the other piece of tubing. Then both the flange and the hole borders are at molten heat the two are brought gently together, being sure of union at all points but avoiding collapse. The union is then smoothed off by alternate heat and blowing as in the simple union except that rotation cannot be effected and each side has to be separately fused. A tendency to attain lumps of glass must be prevented and these smoothed out if they occur, otherwise this will become a weak point in the joint. The three ends of the union are then cut to the desired length. Inasmuch as these joints are generally used for holding rubber tubing a desirable refinement would consist of giving them, prior to making the joint, a double ferrule of glass at the end of each future arm of joint,

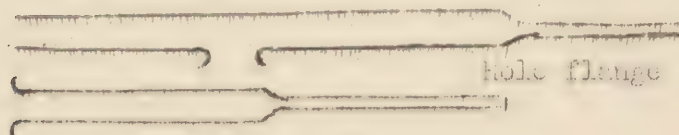
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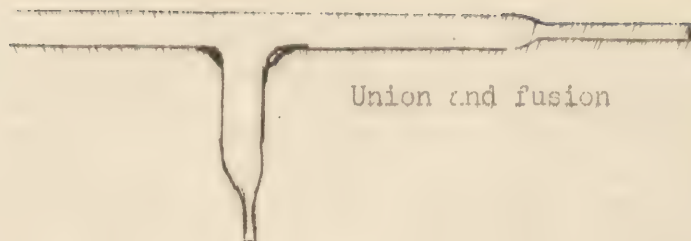
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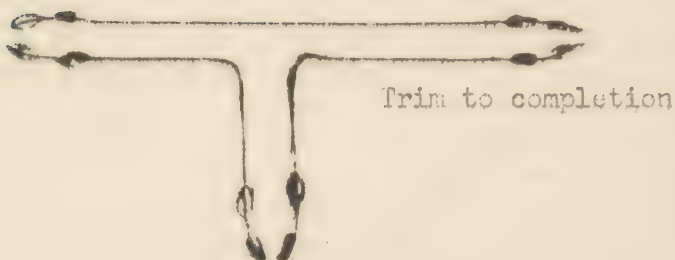
3rd Step



4th Step

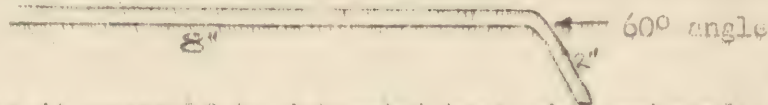


5th Step



#### Exercises in Glass Work:

1. Prepare a "drinking tube" such as used on wards for bed patients.



(try using it yourself to determine best size and angle.)

2. Prepare a "U" tube such as used in laboratory bottles.
3. Prepare a capillary pipette such as will be used for specimen taking.
4. Prepare a glass bulb about the size of a walnut: Round, smooth, even.
5. Prepare a union of a piece of 6 to a piece of 10mm. tubing.
6. Prepare a "Y" or "T" joint.
7. As each of the above is completed to your satisfaction, deliver it to the instructor on a slip bearing your name and a note that you have personally done all parts of the making of the piece.

#### Equipment of this work;

Blast lamp & foot bellows  
Glass tubing; 6 & 10mm.  
File  
Asbestos board  
Wire basket  
Receptacle for waste glass



CHAPTER \_\_\_\_\_

Examination of the Blood.

Finger Puncture.  
Determination of Hemoglobin.  
Red Blood Cell Counting.  
Color Index.  
White Blood Cell Counting.  
Preparation of Blood Smears.  
Differential White Blood Count.  
Characteristics of Stained Cells.  
Schilling's Nuclear Index.  
Reticulocyte Count.  
Platelet Count.  
Coagulation Time.  
Bleeding Time.  
Clot Retraction Time.  
Prothrombin Time.  
Sedimentation Rate.  
Veni Puncture.

## EXAMINATION OF THE BLOOD

The routine laboratory work on blood requires very small amounts of blood, which may be obtained from the finger or ear in adults or from the bottom of the heel in infants. Usually the ball of the middle or ring finger is used. Some of the more technical tests require larger amounts of blood and it is necessary to puncture a vein. This latter procedure will be described in detail near the end of this chapter.

## FINGER PUNCTURE

## Materials

1. A sharp cutting edge is necessary, never use a round needle or pin, because the hole closes too quickly.
  - a. Use a Hagedron needle (cutting edge) or,
  - b. Automatic blood lancet, or,
  - c. Bard-Parker blade (size eleven). This blade may be pushed thru a cork and this cork used to stopper the alcohol bottle. Keep all the cutting blades as clean and shiny as possible.
2. Cotton.
3. Alcohol 70% or Acetone-alcohol equal parts.
4. Clean pipetts and chemically clean slides.

## Procedure.

1. Rub the finger briskly or place hand in warm water to promote blood flow.
2. Clean the finger with the preferred sterilizing solution (alcohol or acetone) and dry. If the finger tip is wet the blood will not form a round drop.
3. Hold the ball of the finger tightly between the operator's thumb and index finger, until the skin color is dark red. Puncture the finger with a firm, quick stroke deep enough so the blood will flow immediately. Do not squeeze the finger after the puncture because this forces tissue juices into the cut and dilates the blood.
4. Wipe off the first drop with dry cotton.
5. Collect a large drop before touching a blood pipette or slide to the drop. Fill the pipettes and make films as indicated.

## DETERMINATION OF HEMOGLOBIN

## Materials

1. Finger puncture equipment.



2. A Tallquist scale which is a sheet of paper with spots of red color on it graded to represent hemoglobin content from 10 to 100%.
3. Absorbent paper supplied in a book with the scale.

#### Procedure

1. Blot a drop of blood with a thin slip of the absorbent paper, set aside to dry. Make the reading as soon as dry.
2. Match against the color standard using a white background, in daylight if possible. This test is the most commonly used, but is very inaccurate and it is only possible to show gross changes. The scale is based on 15.8 grams of hemoglobin per 100 cc of blood equals 100%. Reports are expressed in percentages 90 - 80 - 70 etc.

The Newcomer, Dare, Sahli hemoglobinometers and the Sahli-Hellige Hemometer and the various Photometers which may be used at some stations in the Army will all have printed directions with them. These directions may be consulted and practice with the type of equipment present will be taught at the station. All of the above methods are more accurate than the Tallquist Scale but require more complicated and expensive equipment.

### RED BLOOD CELL COUNTING

#### Materials

1. Cotton or gauze.
2. Lancet for puncture.
3. Water, alcohol and ether for cleaning pipettes.
4. Microscope.
5. Diluting pipette for red blood cells. The Thoma pipette is marked in graduated lines along the capillary bore. The fifth graduation from the tip is marked 0.5 the tenth 1.0 above the bulb is a line marked 101.

In this pipette if blood is drawn to the 0.5 mark and the diluting fluid to 101 the dilution is 1 to 200. The red blood cell pipette often has a red bead in the bulb.

#### 6. Counting Chamber.

The Levy chamber with the Improved Neubauer ruling is the supply table item at the present time. There are other types of ruling and several kinds of chambers; the use of these will be similar to the Levy.

The chamber is a thick glass slide with two central platforms on the surface of each is engraved a series of rulings. The side platforms on which the special cover glass fits are exactly 0.1 mm. higher than the central platforms. When the cover slip is in place there is a space 0.1 mm. deep and the ruled areas have a surface area of 9 sq. mm.

The 4 large corner squares outside the double ruled lines, marked 1, 2, 3, and 4 in the illustration are subdivided into 16 smaller squares.

The central square is divided by double lines into 25 small squares each of which contains 16 smaller squares making a total of 400 squares, see the illustration. Each small square then is  $1/400$  of a sq. mm.

#### 7. Diluting Fluid Hayem's Solution

Sodium chloride	- - - - -	1.0 Gm.
Sodium sulfate	- - - - -	5.0 Gm.
Mercuric chloride	- - - - -	0.5 Gm.
Distilled water	- - - - -	200 cc.

All pipettes and counting chambers should be clean and dry before using and should be cleaned immediately after using. Avoid harsh rubbing or strong solutions on the counting chamber.

(1) With a small piece of cotton scrub the surface of the counting chamber with soap and water, wash with distilled water and air dry, if needed immediately dry on soft gauze.

(2) Draw water through pipettes by suction.

(3) Draw alcohol thru pipettes by suction, this will remove the water.

(4) Draw ether thru the pipettes this will remove the alcohol. Continue the suction for a few seconds to dry the ether. The small bead in the bulb should shake about freely indicating a clean and dry pipette. If pipette becomes plugged thru neglect clean capillary with a horse hair and soak overnight in dilute nitric acid, then clean as above. A water suction pump will make it easier and will save time in pipette cleaning.

#### Procedure

1. Puncture the finger in the usual way.
2. Draw up blood exactly to the 0.5 mark on the red blood cell pipette. Remove any excess on the outside of the tip by wiping on a piece of gauze.
3. Draw up diluting fluid exactly to the mark 101.
4. Shake the pipette in a figure of eight motion for 2 minutes to insure good mixing. Kink the rubber tube and hold the kninked rubber end against the middle finger and the capillary point on the ball of the thumb and shake.
5. Put the cover slip in place on the counting chamber.
6. Blow out 3 - 4 drops, and touch the tip of the pipette to the edge of the platform and allow a thin layer of fluid to flow under the cover glass. If the fluid flows into the troughs or there are bubbles under the Cover glass, clean the counting chamber and try again.
7. Allow the cells to settle for two minutes.
8. Examine under the high dry lens of a microscope.

#### Calculation

Count all the cells in squares A B C D E as illustrated in the drawing of the blood counting chamber. In counting cells in each square



(as A in the illustration) between the double lines; count all cells touching the inner lines on the right and top of the square. Do not count any cells touching the lines on the left and bottom of the square.

#### Example of Calculation

##### 1. By the long method

Block A (see illustration)	16 small squares for example	100
" B	"	98
" C	"	98
" D	"	104
" E	"	100
Total (80 small squares) or 5/25 square mm.	- - - - -	500

To obtain number of cells in 0.1 cu. mm. multiply by 5.

Total in 0.1 cu. mm. (500 x 5) - - - - - 2500

To obtain the number in 1 cu. mm. multiply by 10 or  
(2500 x 10) - - - - - 25000

Dilution 1:200

Total of red blood cells in 1 cu. mm. undiluted blood -  
(200 x 25,000) = 5,000,000

##### 2. By the short method

If the dilution is 1:200 the total cells per cu. mm. can be found by adding four zeros. (500) add 0000 = 5,000,000. The difference between the number of cells in any two blocks should not be more than 15 cells. If this is the case the mixing was not complete or the chamber was dirty.

#### Normal red blood cell counts

Men - - 4,500,000 - 6,000,000 per cu. mm.  
Women - 4,000,000 - 5,500,000 " " "

#### Sources of error

1. Inaccurate dilution - either a bubble or over shooting the mark.
2. Improper placing of the cover slip on the chamber.
3. Over filling the chamber.
4. Not shaking long enough in the pipette.
5. Dirty chamber or pipette.
6. Yeast growing in the diluting fluid.

#### COLOR INDEX.

This means the amount of heoglobin in the average red blood cell of the patient compared with the normal amount.

$$\text{Color index} = \frac{\text{Hemoglobin per cent}}{\text{number of red blood cells per cent}}$$

Considering 5,000,000 per cu. mm. of red blood cells as 100%, the percentage of normal may be obtained by multiplying the first two figures of the red count by two.

#### Example

- |    |                 |           |
|----|-----------------|-----------|
| 1. | Red blood cells | 5,000,000 |
| 2. | Hemoglobin      | 100%      |

$$\text{Color index} = \frac{100}{50 \times 2} = 1$$

A normal color index ranges from 0.85 to 1.15.

### WHITE BLOOD CELL COUNTING

#### Materials

1. Same as for red blood count.
2. White blood cell pipette is similar to the red cell pipette but has a smaller bulb which contains a small white bead. The fifth line on the graduated capillary tube is marked 0.5, the tenth line 1.0, and above the bulb 11.
3. Diluting fluid
 

Glacial Acetic Acid	- - - - -	0.5 cc.
Distilled Water	- - - - -	99.5 cc.

This fluid may be tinted blue for convenience by addition of a drop of 1% gentian violet. This solution should be freshly prepared every two weeks.

#### Procedure

1. Draw blood to the 0.5 mark.
2. Draw diluting fluid to the mark 11, making a dilution of 1:20.
3. Shake as for red blood cells.
4. Discard 3 or 4 drops and fill the counting chamber.
5. Allow the cells to settle.
6. Examine under the low power of the microscope.
7. When doing a complete blood count, shake the red blood pipette in one hand and the white in the other and fill the chamber red on one side and white on the other.

#### Calculation

The white cells are counted in the four large corner squares labelled 1, 2, 3, and 4 in the illustration. Each large square contains 16 smaller squares and has a volume of 0.1 cu. mm. The four squares are counted and divided by 4 to get the average per 0.1 cu. mm.

#### Example of calculation for white blood cells

1. Long method

Square 1 - - - - - 34



## Example of calculation for white blood cells (cont)

Square 2	- - - - -	42
Square 3	- - - - -	38
Square 4	- - - - -	36
Total for 0.4 cu.mm. diluted blood	- - -	<u>150</u>

Total for 0.1 cu.mm. diluted blood  $150 \div 4 = 37.5$

Number of cells in 1 cu.mm. diluted  $37.5 \times 10 = 375$

Dilution 1:20

Total white blood cells per 1 cu.mm.  $20 \times 375 = 7,500$

## 2. Short method

Multiply the total number of cells in 0.4 cu.mm.

$150 \times 50 = 7,500$  white cells per cu.mm.

The difference between the largest and smallest number of cells in any two squares should not exceed 10.

Normal white blood cell count.

5,000 to 10,000 per cu. mm.

Many normal persons have variable counts due to activity, time of day, etc.

Daily counts on a patient should be done at the same time every day.

In certain cases where the white count is very high it may be necessary to use a dilution of 1:100 using the red cell pipette.

In cases where the count is abnormally low make the dilution 1:10 by drawing blood to the 1.0 mark instead of 0.5.

## PREPARATION OF BLOOD SMEARS.

The most necessary part of making a good blood smear is to have chemically clean glass ware.

## Cleaning Slides

## 1. New Slides

- Wash in soapy water and rinse thoroughly with water.
- Place slides in a large beaker of 95% alcohol.
- Polish with a soft lint freecloth (not gauze).
- Flame over a bunsen burner.
- Place in box with clean slip of paper between each slide.

## 2. Dirty slides

- Boil in 5% sodium bicarbonate solution; scrub with soap and water. Place in cleaning solution (potassium bicarbonate-sulfuric acid) for 12 hours.
- Then the same as for new slides.
- Discard all slides that are badly scratched or discolored.

## 3. Cover Glasses

- The same as for slides except do not flame.

### 3. Cover glasses (cont.)

- b. Careful wiping will prevent much breakage, do not use pressure.

In preparing smears it is essential to avoid all unnecessary pressure because of the fragility of the cells.

## The Blood Film

### Materials

1. Equipment for finger puncture.
2. Clean slides free from grease.

### Procedure

1. Puncture the finger.
2. Place one drop of blood on the end of a slide and place the slide on a flat surface.
3. Hold a second slide between the thumb and third finger and place the narrow end, on the slide holding the drop of blood, at a  $30^\circ$  angle.
4. Pull the upper slide until it touches the drop of blood, which then spreads along the narrow end of the top slide. (see illustration in the text).
5. Push the top slide with a firm steady motion toward the opposite end of the bottom slide. The slower the movement the thicker the smear. The greater the angle the thicker the smear.
6. Allow to air dry. In areas where insects are abundant slides must be protected or they will be ruined.

A good smean should be smooth and without waves. The edges should be even and the smear whould not extend to the edges or end of the slide. Labelling may be done by writing on the thicker end of the smear an ordinary lead pencil may be used, when the smear is dry,

The slides should be stained before 24 hours for best results.

### Stain

#### A. Wright's Stain

This stain is used for routine blood smears and seems to be the most satisfactory in ordinary laboratories except for protozoa. The prepared stains purchased by the Army are excellent and easy to prepare if specific directions are followed.

### Materials

1. Chemically clean glass ware.
2. Wright's stain prepared as follows:
 

Wright's powder (supply table item)	- - - - -	0.3 gm.
Glycerine	- - - - -	3.0 cc.
Methyl alcohol absolute (must be acetone free)	- - - - -	97.0 cc.

Put the powder in a dry mortar grind with a pestle, add the glycerine



and grind. Add the methyl alcohol and mix. Allow to stand overnight in a tightly stoppered flask then filter and keep for a few days before use, age improves the stain.

### 3. Buffer solution

Potassium phosphate (monobasic) - - - - - 1.63 Gm.  
 Dibasic sodium phosphate - - - - - 3.2 Gm.  
 Distilled water - - - - - 1000, cc.

### Procedure

1. Cover the dried smear completely with stain for 1 to 3 minutes. This fixes the blood film.
2. Add the buffer solution to the stain drop by drop until a greenish, metallic scum appears on the top. The stain and buffer should cover the slide but none should run off. Determine the time for staining by trial with a series of slides, usually about 2 minutes, but variable with every batch of stain.
3. Wash with water, continue until the slide is a lavender-pink.
4. Allow the slide to stand on edge until dry.
5. Cover with immersion oil and examine under the oil immersion lens. The color of the cells may be varied by changing the pH of the buffer solution. The granules in the neutrophils should stain a lilac color, the eosinophils bright red, the basophils deep blue.

### B. Giemsa Stain

#### Materials.

1. Staining jars (Coplin jar).
2. Giemsa Stain
  - (a) Stock solution
 

Giemsa powder (supply table item) - - - - - 0.5 Gm.  
 Glycerine, dissolve powder in this 1 to 2 hours - 33.0 cc.  
 Methyl alcohol absolute (acetone free) - - - - - 33.00 cc.
  - (b) Dilute stain (ready for use)
 

1 cc. stock solution to 10 cc. distilled water
3. Methyl alcohol.

#### Procedure

1. Fix smear with methyl alcohol 3 to 5 minutes in a Coplin jar.
2. Dry in air.
3. Put in dilute stain for 20 to 30 minutes (Coplin jar).
4. Wash in distilled water.
5. Stand on end to dry.
6. Examine under oil immersion.

This stain is excellent for protozoal staining but is more time consuming than the Wright's stain method. In routine laboratory procedure Giemsa stain is used in staining thick smears for malaria, see section on malaria.

## DIFFERENTIAL WHITE BLOOD CELL COUNT

### Materials

1. Finger puncture and staining equipment.
2. Wright's stain.

### Procedure

1. Prepare films.
2. Stain with Wright's stain.
3. Examine under oil immersion, record each type of white cell seen.

Follow a set pattern in each examination covering the thinner parts of the smear until the proper number of cells are counted.

4. For ordinary laboratory work 100 white cells are counted.

Kolmer and Boerner recommend that the following number of cells be counted depending on the total white blood count.

Total white count under 5,000-----	classify 50 cells
" " " of 5,000-10,000 -----	" 100 "
" " " " 10,000-15,000-----	" 200 "
" " " " 15,000-20,000-----	" 300 "

In the Army laboratories the usual procedure is to count 100 cells classifying them according to the method of Schilling. This method records the percentage of each type of white blood cell. A convenient form for such a count is illustrated below.

	Per cent
Neutrophiles(all types) -----	60-70
Basophiles -----	0 - 1
Eosinophiles -----	1 - 3
Lymphocytes -----	20-35
Monocytes -----	2 - 6

### CHARACTERISTICS OF STAINED CELLS

**I. Red Blood Cells** - Normal red blood cells (erythrocytes) are round, non-granular, non-nucleated cells, the centers of which are less intensely colored than the borders. In various diseases the blood may contain erythrocytes showing the following abnormalities:

Achromia- Pale staining erythrocytes; decreased hemoglobin.

Polychromasia- Many of the erythrocytes take a bluish color rather than a red color.

Anisocytosis- A wide variation in the size of the cells.

Poikilocytosis- Many of the red blood cells are not normally rounded.

Macrocytosis- The average size of the red cells is greater than the normal 7.5 to 8 u.

Microcytosis- The average size of the red cell is smaller than normal.

Stippling- The cells contain a fine dusting of bluish black granules, as seen in lead poisoning.

Reticulocytes- These red cells have feathery, dark blue, irregular fibers within the cell.

Spherocyte- A red cell with the thickness increased and usually the diameter less than 7.5 u. The appearance is that of a sphere or a ball.

Sickle cell- The red cell with an appearance of a sickle or quarter moon. Restricted to an anemia of the negro race.



## II. White Blood Cells:

1. Lymphocytes - These cells are sometimes divided into small, medium and large, in routine counts they are usually classified as small mononuclear lymphocytes.
2. Monocytes - See the color plate for illustration.
3. Granulocytes - See color plate for development and staining reaction of this group of cells.

### SCHILLING'S NUCLEAR INDEX

#### Material

Differential slide equipment.

#### Procedure

Classify the white cells according to headings shown on the illustration of Schilling's Differential Count.

It is necessary to give the doctor a picture of the type of white blood cells present and also the stage of maturity. This is done by dividing the neutrophils into myelocytes, juveniles, staff and segmented. The immaturity of the cells present indicates the increase in the bone marrow output thus throwing young cells into the blood stream.

For illustrations of cells as they develop from the bone marrow see the color plate. The plate is a reproduction from Wright stained cells from Kracke.

### RETICULOCYTE COUNTS

#### Materials:

1. Equipment for finger puncture.
2. Clean glass slides
3. Capillary glass tubes, 2 to 3 mm. outside diameter and about 5 cm long.
4. Supravital stain, prepared by dissolving 1 Gm of brilliant cresyl blue and 5 Gm of Sodium citrate in 100 cc of distilled water.
5. Wright's stain.

#### Procedure:

1. Fill a capillary glass tube half-full of the supravital stain.
2. Place in incubator to dry.
3. When tube is dry, remove from incubator and prepare to stain.
4. Puncture finger.
5. The tube which has been dried is half-filled with blood from the puncture.
6. Tilt tube until all the dried solution is dissolved by the blood.
7. Allow tube to stand for 15 to 30 minutes.
8. Then place the contents of the tube on a clean glass slide and make a thin smear.
9. Counterstain with Wright's stain, and examine under oil immersion.
10. Count 1000 red blood cells noting the number of reticulocytes.

Normal counts: 5 to 10 reticulated red cells per 1000 or .5 to 1%

### PLATELET COUNT

#### Tonio's Smear Method:

#### Materials:

1. Equipment for finger puncture.
2. Equipment for erythrocyte count.
3. Clean slides.
4. 14% Magnesium sulphate solution.
5. Wright's stain.

**Procedure:**

1. Puncture finger and place a drop of 14% magnesium sulphate solution over the puncture before the blood begins to flow.
2. Allow the blood to flow into the drop of 14%  $MgSO_4$  until the proportion is about 1 part of blood to 4 parts of solution.
3. Place this drop on a clean slide and make a thin smear.
4. Wipe away the 14%  $MgSO_4$  solution and do a red blood cell count.
5. Stain smear with Wright's stain and examine under oil immersion.
6. Count 1000 red blood cells noting the number of platelets.

Calculation: NO. of platelets  $\times \frac{\text{red blood cell count}}{1000} =$  number of  
per cubic mm.  
of blood.

**COAGULATION TIME****I. Slide method****Material:**

1. Finger puncture equipment.
2. Clean slide.
3. Needle.
4. Watch.

**Procedure:**

1. Clean finger with alcohol and make routine puncture.
2. Place a few drops of blood on slide.
3. At  $\frac{1}{2}$  minute intervals draw a needle slowly thru the blood drop. When a fine thread (fibrin) can be picked up by the needle point of coagulation has begun.
4. The time between the flow of blood and the formation of fibrin is coagulation time.

**II. Capillary tube method**

Draw out soft glass tubing into a capillary pipette, over a wing top bunsen burner. After the finger puncture fill the tube, then at  $\frac{1}{2}$  minute intervals break off 3-4 mm. of the tube. If the fibrin strings out at the broken end coagulation has begun. Normal coagulation time is 2 to 8 minutes.

**CLOT RETRACTION TIME****Materials**

1. Equipment for venipuncture.
2. Test tube or a blood vacuum tube.
3. Incubator.
4. Watch.

**Procedure**

1. Place 2 cc. of blood in a dry test tube.
2. Incubate at 37 degrees C. for 24 hours.
3. Observe at the end of each hour for 6 hours and at intervals of 6 to 12 hours thereafter. Under these conditions the clot normally retracts completely within 18 to 24 hours after it is formed. Delay in retraction, or failure of the clot to retract, is usually associated with a decrease in platelets.



## Wintrobe and Landsberg Method

## Material

1. Special hematocrit tube, which is 100 mm. long, has a uniform bore of 3 mm. and has a flat bottom. It is graduated on one side from 0 to 10 cm. in 1 mm. divisions with white numerals, and on the other side from 10 cm. to 0 in 1 mm. divisions with red numerals.
2. Anticoagulant, which is a mixture of 6 mg of Ammonium oxalate and 4 mg of potassium oxalate for each 5 cc. of blood. Tubes for the blood may be prepared by placing the proper amount of the oxalates in solution, and then evaporating the water by placing the tubes in a dry heat oven.
3. Venipuncture equipment.
4. 5 cc sterile syringe.

## Procedure

1. Withdraw 5 cc. of venous blood in a dry sterile syringe and place in a tube or bottle containing the anticoagulant described above.
2. With a capillary pipette fill a Wintrobe hematocrit tube to the 10 cm mark.
3. Place the filled tube in exactly vertical position, at room temperature, and observe the point on the mm. scale to which the corpuscles fall during exactly one hour.
4. Centrifugalize the tube until packing of red cells is complete; then read the volume of packed red cells.
5. Correct the rate according to the volume of cells by referring to the chart for correction on page 13, as follows:

Find the horizontal line which represents the sedimentation in mm. for one hour. Follow this across the chart until it intersects the vertical line which represents the blood cells volume per cent. Follow the nearest curved line until it intersects the heavy line at 45 cc. per 100 cc. Then at the point of intersection read the value on the horizontal line for the corrected sedimentation rate. Maximal normal range is from 0 to 9 mm for men, and 0 to 20 mm. for women, in one hour.

VENIPUNCTURE  
(For Blood Culture)

## Material

1. Luer syringe, 10 cc., sterile.
2. Needle, 20 gauge, sterile.
3. Flask of appropriate culture medium.
4. Tincture of iodine.
5. Alcohol, 70%
6. Tourniquet.
7. Cotton or gauze pledgets, sterile.
8. Alcohol lamp, if no gas burner is available.

## Procedure

1. Thoroughly cleanse skin over the vein and surrounding area of the arm for about three inches, with alcohol.
2. Paint over the vein with iodine and leave on for 2 or 3 minutes.
3. Light alcohol lamp or burner.
4. Unwrap syringe and insert plunger into barrel. Do not touch inside of barrel or shaft of plunger.
5. Remove plug from needle tube and flame mouth of tube.



5. Puncture the skin a little to one side of the vein and parallel to it. If the needle is sharp, this can be done with one quick motion and is not at all painful.
6. Turn the needle point slightly toward the vein and enter with a quick, short stab. If you turn the point too squarely toward the vein you risk puncturing both walls.
7. When you have collected 10 to 15 cc. of blood, loosen the tourniquet.
8. Press a pledget of cotton soaked with alcohol or acetone over the puncture and withdraw the needle quickly, maintaining the pressure until the bleeding, if any, has stopped.

### Care of Needles and Syringes

1. As soon as the blood sample is taken, shake as much of the blood from the needle as possible and drop it into a beaker of water to take the blood. On return to the laboratory, clean thoroughly with cold water and dry by forcing alcohol by ether through the bore. Never put a wet needle away, as rust is dangerous.
2. Replace the stylet, leaving the loop of wire outside the point for the protection of the latter. Slide the needle, point down, into a Wasserman tube, plug with cotton and sterilize by dry heat or in the autoclave.
3. Sharpening the needle is best done on the finest grade of emery cloth stretched on a flat surface. Finish on a fine blue water stone. Even the finest grade of emery or carborundum, if used alone will leave a slight saw edge that may cause too much pain.
4. Syringes must never be left with the plunger in the barrel after use, no matter what has been in the syringes. Always wash out the syringe with water immediately after use and leave the plunger out until both it and the barrel have been carefully dried. Once a plunger has been "frozen" in the barrel, it may not be possible to remove it. Often the best way to free it is to force cold water through the neck of the barrel against the head of the plunger, using another syringe with a needle small enough to insert into the neck. Warming the barrel in hot water may loosen it. Soaking in cold water for several days may be necessary. Never use force.
5. To sterilize, wrap the plunger and barrel separately in gauze, with an outer wrapping of heavy paper. Secure the wrapping with a turn or two of ordinary twine, tied in a slip knot to expediate unwrapping.

### Cautions

1. Certain dangers and discomforts to the patient must be avoided. These are (a) Infection, (b) Injury to the vein wall, (c) hematoma, (d) Needless pain.
2. Infection is due to carelessness. Be certain your needles and syringes are sterile. Never touch the shaft of the needle to anything that is not sterile before taking the blood.
3. Injury to the vein wall may cause a clot to form on the wall. This may break free in the blood stream and death may result. Causes: Dull or rusty needles, too much movement of the needle point while in the vein, passing of stylet through the needle while still in the vein in the attempt to free the needle from clots. Never pass the stylet through needle before withdrawal. If clots plug the needle, withdraw it and try and try the other arm.



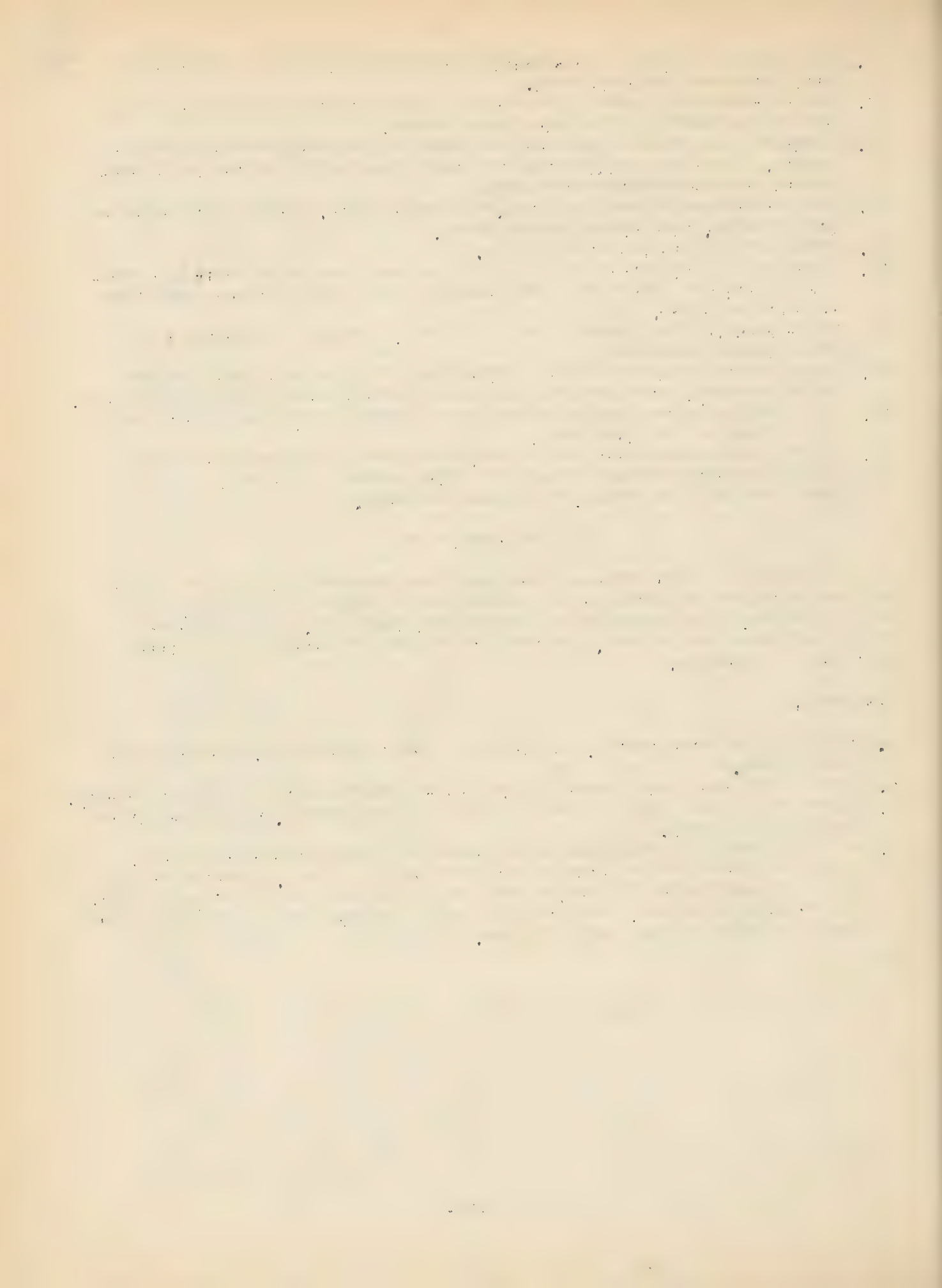
6. Insert neck of syringe into mouth of tube and tilt tube so that needle will slide down over the neck.
7. Remove syringe and needle and set the needle firmly on the neck, being careful to touch only the hub of the needle.
8. Flame both the needle point and the mouth of the tube that contained the needle. Cover the needle with the flamed tube and set aside while completing the preparation of the arm.
9. Apply tourniquet above the elbow, not too tightly. If the vein does not distend well, have patient clench fist.
10. Sponge off the iodine with alcohol.
11. Puncture the skin with needle a little to one side of the vein and parallel to it; then enter the vein from that side about half an inch above the skin puncture.
12. After securing the desired amount of blood, loosen the tourniquet and have patient open fist.
13. Press an alcohol-soaked pledget firmly over the puncture and withdraw needle quickly. Have patient flex elbow tightly to hold pledget in place.
14. Open flask and flame mouth thoroughly, holding the syringe near but not in the flame at the same time.
15. Insert needle into flask and force blood directly into culture medium without touching sides of flask with either needle or blood. Flame neck of flask again, replug and incubate.

#### For Other Purposes

In taking the large number of routine blood samples required for other purposes which do not demand sterile blood, it is simpler to use only the sterile needle, as so many syringes are seldom available. The method is simple and takes little time, provided directions are followed and a little patience is exercised.

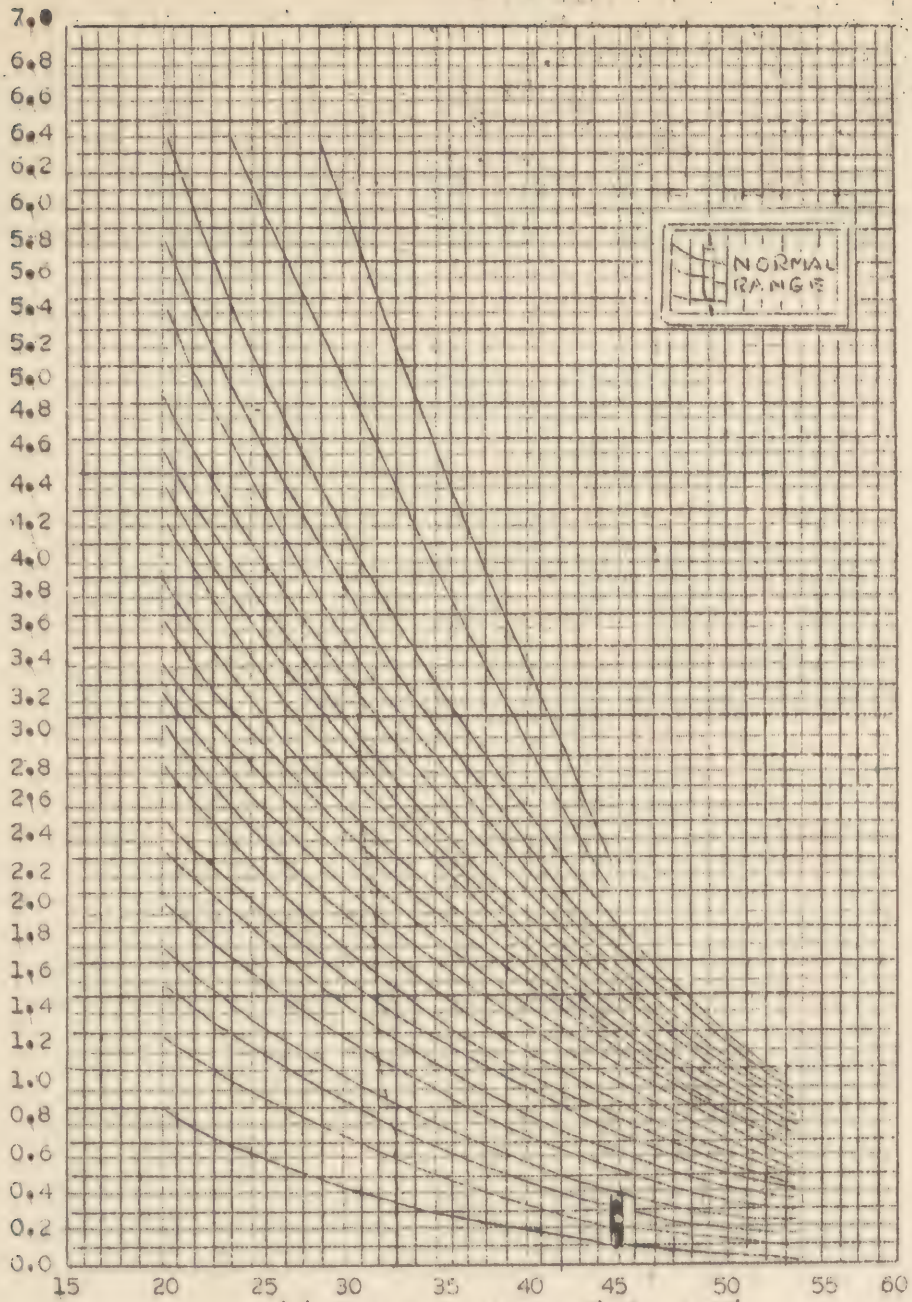
#### Procedure;

1. Swab the site with iodine, followed by alcohol or acetone. Acetone alone may be used.
2. Tighten the tourniquet about the upper arm enough to dilate the vein firmly.
3. Remove the needle from its tube and take the stylet out, being careful to touch only the hub.
4. Hold the needle tightly between the thumb and index finger at the hub. Insert the tube for the blood specimen below the needle, grasping it with the third and fourth fingers, so that the hub of the needle is just within the mouth of the tube. This is much easier to accomplish if the patient's arm is allowed to hang straight down.





MILLIMETERS PER MINUTE



HEMATOCRIT

PER CENT CELLS

4. Hematoma (blood tumor) is often very painful and may become infected. Causes: Too large a needle in a delicate vein, withdrawal of the needle before the tourniquet is loosened, making insufficient pressure over the puncture after withdrawal.
5. Needless pain is often due to excess of care and slowness in making the puncture. Dull needles most often cause it. A sharp 17 gauge causes less pain than a dull 20 gauge. Remember that those ill enough to be in a hospital may be greatly set back by even a slight painful shock, especially if many blood tests have to be taken. It is always better to make patients lie down or sit in comfort while blood is being taken.



VENIPUNCTURE.1. For Blood Culture.

## Materials:

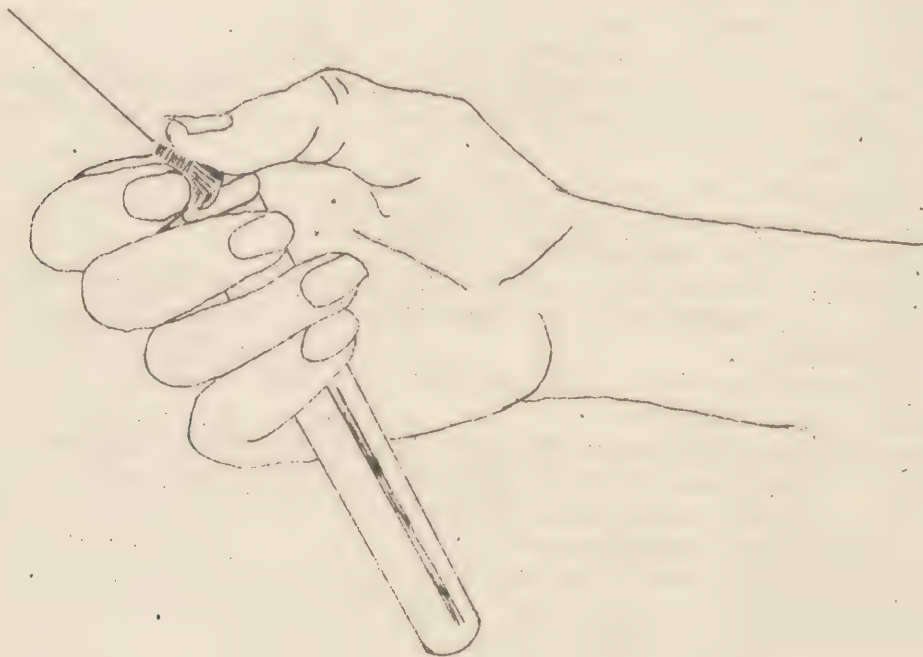
Luer syringe, 10 cc., sterile.  
Needle, 20 gauge, sterile.  
Flask of appropriate culture medium.  
Tincture of iodine.  
Alcohol, 70%.  
Tourniquet.  
Cotton or gauze pledgets, sterile.  
Alcohol lamp if no gas burner is available.

- a. Thoroughly cleanse the vein and surrounding area of the arm for about three inches, with alcohol.
- b. Paint over the vein with iodine and leave on for 2 or 3 minutes.
- c. Light alcohol lamp or burner.
- d. Unwrap syringe and insert plunger into barrel. Do not touch inside of barrel or shaft of plunger.
- e. Remove plug from needle tube and flame mouth of tube.
- f. Insert neck of syringe into mouth of tube and tilt tube so that needle will slide down over the neck.
- g. Remove syringe and needle and set the needle firmly on the neck, being careful to touch only the hub of the needle.
- h. Flame both the needle point and the mouth of the tube that contained the needle. Cover the needle with the flamed tube and set aside while completing the preparation of the arm.
- i. Apply tourniquet above the elbow, not too tightly. If the vein does not distend well, have patient clench fist.
- j. Sponge off the iodine with alcohol.
- k. Puncture the skin with needle a little to one side of the vein and parallel to it; then enter the vein from that side about half an inch above the skin puncture.
- l. After securing the desired amount of blood, loosen the tourniquet and have patient open fist.
- m. Press an alcohol-soaked pledget firmly over the puncture and withdraw needle quickly. Have patient flex elbow tightly to hold pledget in place.
- n. Open flask and flame mouth thoroughly, holding the syringe near but not in the flame at the same time.
- o. Insert needle into flask and force blood directly into culture medium without touching sides of flask with either needle or blood.  
Flame neck of flask again, replug and incubate.

2. For Other Purposes.

In taking the large number of routine blood samples required for other purposes which do not demand sterile blood, it is simpler to use only the sterile needle, as so many syringes are seldom available. The method is simple and takes little time, provided directions are followed and a little patience is exercised.

- a. Swab the site with iodine, followed by alcohol or acetone. Acetone alone may be used.
- b. Tighten tourniquet about the upper arm enough to dilate the vein firmly.
- c. Remove the needle from its tube and take the stylet out, being careful to touch only the hub.
- d. Hold the needle tightly between the thumb and index finger at the hub. Insert the tube for the blood specimen below the needle, grasping it with the third and fourth fingers, so that the hub of the needle is just within the mouth of the tube (see illustration). This is much easier to accomplish if the patient's arm is allowed to hang straight down.
- e. Puncture the skin a little to one side of the vein and parallel to it. If the needle is sharp, this can be done with one quick motion and is not at all painful.
- f. Turn the needle point slightly toward the vein and enter with a quick, short stab. If you turn the point too squarely toward the vein you risk puncturing both walls.
- g. When you have collected 10 to 15 cc. of blood, loosen the tourniquet.
- h. Press a pledget of cotton soaked in alcohol or acetone over the puncture and withdraw the needle quickly, maintaining the pressure until the bleeding, if any, has stopped.



#### Care of Needles and Syringes.

As soon as the blood sample is taken, shake as much of the blood from the needle as possible and drop it into a beaker of water to lake the blood. On return to the laboratory, clean thoroughly with cold water and dry by forcing alcohol followed by ether through the bore. Never put a wet needle away, as rust is dangerous.



Replace the stylet, leaving the loop of wire outside the point for the protection of the latter. Slide the needle, point down, into a Wassermann tube, plug tube with cotton and sterilize by dry heat or in the autoclave.

Sharpening the needles is best done on the finest grade of emery cloth stretched on a flat surface. Finish on a fine blue water stone. Even the finest grade of emery or carborundum, if used alone, will leave a slight saw edge that may cause too much pain.

Syringes must never be left with the plunger in the barrel after use, no matter what has been in the syringe. Always wash out the syringe with water immediately after use and leave the plunger out until both it and the barrel have been carefully dried. Once a plunger has been "frozen" in the barrel, it may not be possible to remove it. Often the best way to free it is to force cold water through the neck of the barrel against the head of the plunger, using another syringe with a needle small enough to insert into the neck. Warming the barrel in hot water may loosen it. Soaking in cold water for several days may be necessary. Never use force.

To sterilize, wrap the plunger and barrel separately in gauze, with an outer wrapping of heavy paper. Secure the wrapping with a turn or two of ordinary twine, tied in a slip knot to expedite unwrapping.

### Cautions.

Certain dangers and discomforts to the patient must be avoided. These are (a) Infection, (b) Injury to the vein wall, (c) Hematoma, (d) Needless pain.

Infection is due to carelessness. Be certain your needles and syringes are sterile. Never touch the shaft of the needle to anything that is not sterile before taking the blood.

Injury to the vein wall may cause a clot to form on the wall. This may break free in the blood stream and death may result. Causes: dull or rusty needles, too much movement of the needle point while in the vein, passing of the stylet through the needle while still in the vein in the attempt to free the needle from clots. Never pass the stylet through the needle before withdrawal. If clots plug the needle, withdraw it and try the other arm.

Hematoma (blood tumor) is often very painful and may become infected. Causes: Too large a needle in a delicate vein, withdrawal of the needle before tourniquet is loosened, making insufficient pressure over the puncture after withdrawal.

Needless pain is often due to excess of care and slowness in making the puncture. Dull needles most often cause it. A sharp 17 gauge causes less pain than a dull 20 gauge. Remember that those ill enough to be in a hospital may be greatly set back by even a slight painful shock, especially if many blood tests have to be taken. It is always better to make patients lie down or sit in comfort while blood is being taken.





## BLOOD GROUPING

Instructions have been issued for the typing the blood of every man in the Arm. It is impractical to procure enough human sera of Groups A and B to accomplish this. Powdered anti-sera must therefore be used for the purpose. This is made by immunizing rabbits to human red cells of groups A and B. Sera from these immunized rabbits are dried, powdered and mixed with sucrose. They are then standardized to uniform strength and are more rapid in action than the human sera.

The performance and the reading of the test with these anti-sera differ from those using human sera. A new technique must be outlined.

This technique, given below, is adapted from Circular Letter No. 70, S.G.O., July 14, 1941.

### 1. Materials.

Anti-A, powdered rabbit serum (colored with methylene blue).

Anti-B, powdered rabbit serum (colored with eosin).

Physiologic salt solution.

Small test tubes (Kahn tubes are suitable).

Microscopic slides.

Wooden toothpicks or wooden applicators.

Wax pencil.

### 2. The Test.

a. Carefully cleanse the finger of the man to be tested (with either alcohol or acetone) and puncture with a sterile needle. Collect three or four drop of the blood in a small test tube containing 1.0 cc of physiologic salt solution. Mix blood and salt solution by gentle shaking. This makes a cell suspension of about 10% strength.

b. With a wax pencil draw a line down the middle of a clean glass slide and label one end "A" and the other end "B." With a clean medicine dropper place a large drop of the cell suspension in the center of each of the two ends "A" and "B." Then, with the broad end of the blue tipped toothpick, dip up a small mound (about 2 mm. long) of group A powdered anti-serum (colored with methylene blue) and add this to the drop of cell suspension on the "A" side of the slide. Likewise, using the red tipped toothpick, add a similar amount of group B powdered anti-serum (colored with eosin) to the drop of cell suspension on the "B" side of the slide. Mix the preparations thoroughly by stirring each with the unused end of a clean toothpick or applicator. Allow the preparations to stand from one to two minutes and read the result.

CAUTION. When a medicine dropper is used to transfer cell suspensions in a series of tests great care must be taken to wash it out with salt solution after each test, so that no blood cells from the previous test will be carried over to the next. After blowing out the contents of the dropper, fill the barrel full of fresh saline and expel it into a beaker for that purpose. Repeat this three times. If the salt solution accidentally becomes mixed with cells from the dropper, discard and use fresh solution.

### 3. The Reading.

a. If no agglutination occurs in either A or B anti-sera, the blood being tested belongs to Group "O" (the universal donor.)

b. If agglutination occurs in the anti-A serum only, the blood belongs to group "A."

c. If agglutination occurs in the anti-B serum only, the blood belongs to group "B."

d. If agglutination occurs in both anti-sera A and B, the blood belongs to group "AB."



Technicians who are used to making the test with straight human sera must be careful not to confuse the two methods. The readings of groups A and B are reversed and the coloring of the anti-sera is the opposite of that previously used by the Army with straight human sera.

#### 4. Recording.

The medical officer will be responsible for the recording of the results of the blood grouping as follows:-

a. A record will be made, using the Graphotype machines, by stamping clearly on the individual's identification tag, in spaces 17 and 18 of line 2, the blood group to which he belongs. The symbols "O," "A," "B" or "AB" will be used as indicated.

b. The careful and accurate recordings of this information on the identification tag is imperative, as error may result in death by leading to the use of the wrong type of blood for transfusion. For this reason the result recorded on the tag must be verified by an officer of the Medical Corps, by checking it with the individual's blood grouping test.

c. Using the model 70 Addressograph, an additional record will be made in the case of the enlisted men on the individual's Service Record, A.G.O. Form 24, under the heading "Other Vaccinations," and for others on the Immunization Register, M.D. Form 81, in the blank space provided for the record of injections of "Stimulating doses of tetanus toxoid."

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### A SUGGESTED PROCEDURE FOR DETERMINING AND RECORDING THE BLOOD TYPES OF MILITARY PERSONNEL.

The test to be used in determining the blood types of military personnel is so simple that elaborate laboratory facilities will not be required. In small posts or isolated detachments the tests can be done by the Surgeon with a few enlisted assistants. In large organizations it is suggested that they may be performed expeditiously by using one or more "blood typing teams" composed of Medical Department personnel as outlined below. The individuals to be typed should be required to report for the examination at some convenient central place and to bring with them their identification tags. Each "blood typing team" should be supplied with a Graphotype machine for recording the results of the tests on the tags. It is estimated that a single team should be able to determine the blood types and record the findings for 400 men in an eight-hour day.

#### ORGANIZATION OF A BLOOD TYPING TEAM.

The men whose blood is to be typed should report by organizations to a place designated by the officer in charge of the blood typing team, after which they will pass through the following "stations" in sequence:

- STATION 1. At this station an enlisted man seated at a table, adding to small glass vials 1.0 cc amounts of physiological salt solution by means of a calibrated medicine dropper, will give one tube of salt solution to each individual to be tested.
- STATION 2. At this station two (2) enlisted men, taking alternate individuals, will collect from the finger of each individual four drops of blood, adding this directly to the vial of salt solution. The vial will be returned to the individual who will carry it to Station 3.



STATION 3. At this station two (2) enlisted men, taking alternate individuals, will prepare the tests by placing with a clean medicine dropper (see CAUTION, above) a drop of the blood cell suspension on each of the two labeled ends of the glass slide, and then adding the dried anti-sera A and B respectively as outlined above in the technique for the test. The slide containing the individual's blood grouping test together with his vial will be returned to him, and he will then carry them to Station 4 where the results will be read and recorded.

STATION 4. At this station one (1) enlisted man will read the result of the test to the Graphotype operator who will record the result on the individual's identification tag. The individual will then carry his vial of blood cells, his blood grouping test slide, and his identification tag to the next station.

STATION 5. Here the Medical Officer will verify the result of the test and check this against the record made on the identification tag in order to prevent errors in recording.

NOTE. It seems obvious that some mistakes will have been recorded on the tags before this check has been made. This would entail making out a good many new tags in the aggregate. This might be prevented by a check prior to stamping the tags. If available another medical officer might well be used for this purpose, or the medical officer already detailed might make the check with the enlisted man at Station 4.

STATION 6. Here the slides and vials will be turned in to two (2) enlisted men who will wash and dry them and return them to Station 1.





## PEROXIDASE STAIN

(Washburn's Modification of Goodpasture's Method)

### MATERIALS:

#### Solution # 1

Benzidine base ..... 0.3 gms  
Basic fuchsin ..... 0.3 gms  
Sodium nitroprusside, saturated aqueous solution, ..... 1.0 cc  
Ethyl alcohol, 95% ..... 100.0 cc

Dissolve the benzidine and fuchsin in the alcohol in the order named. Then add the nitroprusside solution. A small precipitate may form at the bottom of the container but does not interfere with the staining qualities of the solution. This solution will keep for 8 to 10 months.

#### Solution # 2

Hydrogen peroxide ..... 0.3 cc  
Tap water ..... 25.0 cc

This solution will keep for about 2 days.

### PROCEDURE:

1. Flood the smear with 10 drops of Solution # 1 for 1 to  $1\frac{1}{2}$  minutes.
2. Without pouring off the stain, add 5 drops of Solution # 2 and allow to stand 3 to 4 minutes.
3. Wash in tap water for  $\frac{1}{2}$  to 1 minute.
4. While wet, flood with 95% ethyl alcohol. Keep alcohol on until smear is completely decolorized and no more pink is visible. Several changes of alcohol may be necessary.
5. Wash thoroughly in tap water and dry in air.
6. Counterstain with Wright's Stain.

With this stain, all leucocytes and erythrocytes will appear as they usually do with Wright's stain. However, the granules of all granulocytes with the exception of the myeloblasts will be stained black. Monocytes may or may not show black granules. The azurophilic granules of lymphocytes do not stain black.

## BLEEDING TIME

Bleeding time may be defined as the time required for the cessation of hemorrhage when blood flows from a cut in the skin of approximately measured depth and length.

### Duke's Method:

#### MATERIALS:

1. Equipment for finger puncture.
2. Filter paper.
3. Stop watch.

#### PROCEDURE:

1. Incise the skin or make a deep cutting puncture in the finger or lobe of the ear.
2. Note the time the first drop appears.

3. Blot with filter paper each drop as it flows out or blot every half minute taking care not to rub the incision with the filter paper.

4. The interval between the first drop and the last is considered the bleeding time.

The normal bleeding time by this method is 1 to 3 minutes.

#### FRAGILITY TEST

##### Sanford's Method

##### MATERIALS AND EQUIPMENT:

1. Equipment for venipuncture. Syringe and needle should be dry.
2. Twelve small test tubes of approximately the same diameter are placed in a rack and labelled 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15 and 14.
3. Capillary pipette or syringe and needle (in order to have all drops same size).
4. 0.5% solution of NaCl made volumetrically, Made with fresh distilled water.

##### PROCEDURE:

1. Using the capillary pipette, drop as many drops of 0.5% NaCl into each test tube as indicated by the number on the tube. A syringe and needle may be used in place of the pipette but in either case hold at the same angle to insure equality in the size of the drops.
2. With the same pipette, add drops of distilled water to every tube except the first, of sufficient number to make a total of 25 drops in each tube. Invert and mix. The percentage strength of sodium chloride in each tube is equal to the number of the tube multiplied by 0.02.
3. Withdraw 1 to 1.5 cc. of blood from a vein and place one drop of blood in each tube. Mix by inverting and allow to stand at room temperature for 2 hours.
4. Determine point in which hemolysis begins and point where hemolysis is complete.
5. Prepare a similar set of tubes, using the blood of a normal individual for testing the accuracy of the solutions. This control must be made with every test.

Normal blood shows beginning hemolysis in 0.44 to 0.42 per cent salt solution and complete hemolysis from 0.34 to 0.32 per cent. A positive test should be repeated and higher concentrations of hypotonic NaCl used, if indicated, for additional tests.



## CLEANING OF SEROLOGICAL GLASSWARE

Serum & Kahn Tubes & Miscellaneous  
Flasks, Beakers, Etc.

1. Rinse under full force of tap as soon as possible after use.
2. Boil 1 hour using 1/3 cake of mild white soap chipped. Flasks & beakers need not be boiled. Thorough brushing with warm water and soap is sufficient.
3. Rinse six times under full force of tap.
4. Soak in tap water over night.
5. Rinse with distilled water.
6. Dry in dry-wall oven two hours.

Pipettes

1. Submerge in water immediately after use.
2. Rinse under full force of tap.
3. Soak in bichromate solution 18 to 24 hours.  
(Have found specimen jar most practical for this)
4. Pour off bichromate & rinse 6 times under full force tap.
5. Soak in tap water over night.
6. Rinse with distilled water.
7. Dry in dry-wall oven two hours

Colloidal Gold Tubes, Flasks,  
Beakers, Etc.

1. Rinse under full force tap.
2. Submerge in Gold Solvent for one hour.
3. Pour off Gold Solvent & rinse 3 times under full force tap.
4. Submerge in bichromate solution 18 to 24 hours. Flasks & beakers may be brushed with soap instead.
5. Rinse 6 times under full force tap.
6. Soak in tap water over night.
7. Rinse with distilled water.
8. Dry in dry-wall oven two hours.
9. Keep colloidal gold tubes separate

Bichromate Solution

Sodium Bichromate	320 grams
H <sub>2</sub> SO <sub>4</sub> Commercial	450 cc
or fuming	420 cc
Tap Water	3000 cc

Gold Solvent (Aqua Regia)

H Cl	800 cc
HNO <sub>3</sub>	400 cc
Tap Water	1000 cc





## STANDARD KAHN TEST PROCEDURE

1. Separate serum from clot and centrifuge until entirely free of cells.
2. Inactivate serum in water bath (56 degrees) for 30 minutes.
3. Mix Kahn antigen by placing 1cc. of antigen in a mixing vial and the required amount of .85% salt solution (as indicated by titer on antigen bottle) in another vial, then pour salt into antigen and quickly pour from one vial to the other 12 times. Allow to stand for ten minutes.
4. The Standard Kahn Test is a three tube test. The tubes are placed in the rack one in back of another three deep.
5. After the antigen has stood for ten minutes it is pipetted into the bottom of the tubes in the following varying amounts:

Back tube-----	.0125 cc
Middle tube-----	.025 cc
Front tube-----	.05 cc

A Kahn antigen pipette (total capacity .25cc) is used to deliver the antigen.
6. The inactivated serum of the patient is then added to the tubes containing the antigen, 0.15cc to each tube.
7. Controls.

Three controls are set up at the same time the test is run. The same amounts of antigen ( paragraph 5. ) are used.

  - a. Control one--0.15cc of .85% salt solution is added to antigen in each of the three tubes.
  - b. Control two--0.15cc of known positive serum is added to antigen in each of the three tubes.
  - c. Control three--0.15cc of known negative serum is added to antigen in each of the three tubes.
8. All tubes are shaken for three minutes.
9. Salt solution is added to the tubes in the following amounts:

Back tube-----	0.5cc
Middle tube-----	0.5cc
Front tube-----	1.0cc
10. All tubes are shaken enough to mix the salt solution and antigen-serum mixture thoroughly.
11. Tests are then read.





## Quantitative Kahn Test Procedure

To be done only on sera that show a positive reaction with the Standard Kahn test.

1. Separate serum from clot and centrifuge until entirely free of cells.
2. Inactivate serum in water bath ( 56 degrees C ) for 30 minutes.
3. Mix Kahn antigen by placing 1cc. of antigen in a mixing vial and the required amount of .85% salt solution ( as indicated by titer on antigen bottle) in another vial, then pour salt into antigen and quickly pour from one vial to the other 12 times. Allow to stand for ten minutes.
4. While antigen is aging for ten minutes dilutions are made of the serum as follows: ( six tubes are used.)
 

Tube 1----	.6cc salt solution	+	.4cc serum
Tube 2----	.5cc salt solution	+	.5cc of mixture from tube 1
Tube 3----	.5cc salt solution	+	.5cc of mixture from tube 2
Tube 4----	.5cc salt solution	+	.5cc of mixture from tube 3
Tube 5----	.5cc salt solution	+	.5cc of mixture from tube 4
Tube 6----	.5cc salt solution	+	.5cc of mixture from tube 5
5. Six Kahn tubes are placed in the Kahn rack in a row, then .025 cc of antigen suspension is pipetted into the bottom of each of these tubes.
6. 0.15cc of each of the serum dilutions is then added to the corresponding Kahn tube, beginning with tube 6. That is----
 

.15cc	from serum dilution tube 6	into Kahn tube #6
.15cc	from serum dilution tube 5	into Kahn tube #5 ---etc.
7. All tubes are shaken for three minutes.
8. 0.5cc of salt solution added to each tube.
9. Shake rack and read results.





### Spinal Fluid Kahn Procedure

1. Centrifuge spinal fluid until free of cells.
2. Pipette 1.5 cc of clear spinal fluid into centrifuge tube.
3. Add 1.5 cc of saturated ammonium sulfate solution.
4. Place in 56 degree water bath for 15 minutes. (Globulin can be read at this point)
5. Centrifuge at high speed for ten minutes. The precipitated globulin will be packed in the bottom of the centrifuge tube. Pour off the supernatant fluid and drain by inverting the tube on a clean towel for several minutes.
6. Add .15 cc of .85% salt solution and shake gently until the precipitate is dissolved.
7. Mix antigen by placing 1. cc of antigen in a mixing vial and the required amount of .85% salt solution (as indicated by titer on antigen bottle) in another vial, then pour salt into antigen and quickly pour from one vial to the other 12 times. Allow to stand for ten minutes.
8. Pipette .01 cc of antigen suspension into the bottom of a Kahn tube.
9. Pipette .15 cc of the globulin solution from the centrifuge tube into the Kahn tube which contains the .01 cc of antigen suspension.
10. Shake for three minutes, add .5 cc of salt solution, read results.

### Quantitative Kahn for Spinal Fluid

1. Centrifuge spinal fluid until free of cells.
2. Pipette 3 cc of clear spinal fluid into centrifuge tube.
3. Add 3 cc of saturated ammonium sulfate solution.
4. Incubate, centrifuge, drain and dilute antigen as above.
5. Add .3 cc of salt solution and dissolve precipitate as above.
6. While antigen is aging dilutions are made of the globulin solution— as follows: (Five tubes are used)
  - Tube 1.—This is the .3 cc of globulin solution in the centrifuge tube.
  - Tube 2.—0.6 cc salt solution / .15 cc from tube 1
  - Tube 3.—0.4 cc salt solution / .4 cc from tube 2
  - Tube 4.—0.1 cc salt solution / .2 cc from tube 3
  - Tube 5.—0.2 cc salt solution / .2 cc from tube 3

7. This is a 5 tube test. Place 5 Kahn tubes in the rack in a row.
8. Pipette antigen that has stood for 10 minutes into Kahn tubes .01 cc into the bottom of each tube.
9. Pipette .15 cc of dilutions of globulin solution into tubes containing the antigen beginning with the last tube. That is—  
    .15 cc from dilution tube 5 into Kahn tube #5  
    .15 cc from dilution tube 4 into Kahn tube #4—etc.
10. Shake three minutes, add .5 cc of salt solution, read results.



## A SIMPLE AND ACCURATE METHOD FOR MAKING

## COLLOIDAL GOLD SOLUTION.

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The following formula appears to have taken the worry and uncertainty out of a formerly worrying and doubtful procedure. It is a modification of the method described by Porowskaja; Zeitschrift fur Immunitatsforschung und Experimentelle Therapie, May 8, 1934, vol. 82, no. 3, p. 178.

Its advantages are speed and simplicity in preparation, permanence and adequate sensitivity. It may be made up in amounts as small as 100 cc. in laboratories where these tests are infrequently made. In routine use at the Serological Laboratory of the Army Medical School it has been found that the color changes are clear-cut, easily read and closely confirmatory of the clinical findings. Occasional unexpected reactions have been borne out by further clinical developments.

The Method.

## 1. Glassware, etc.

Erlenmeyer flasks,		
2000 cc		1.
250 or 500 cc		1.
Graduates		
1000 cc		1.
50 cc		1.
Pipettes		
10 cc	Several.	
1 cc	Several.	
Thermometer, 100 degree C.		1.
Beakers, 250 cc	Several.	
4-burner Bunsen		1.

The glassware should be chemically clean and should be rinsed inside and out with double-distilled water before using. Pyrex glass is not essential except in the flask used for boiling. Quicker heating without wire gauze may thus be attained and is important. It is also better to store the completed solution in pyrex but not absolutely necessary.

## 2. Reagents.

(1). Double-distilled water.

(2). 1% solution of gold chloride. (Note. Merck's chloride, which should be used, comes in 15 grain ampoules, therefore the contents of one ampoule dissolved in 97.2 cc. of double-distilled water makes a 1% solution). Be sure to soak off the label and all paste in warm water and rinse thoroughly in double-distilled water before breaking. Take care that none of the chloride is lost in breaking. Place the halves of the ampoule with their contents in beaker containing 97.2 cc. of double-distilled water and stir thoroughly to insure complete and even solution of the chloride.

(3). 1% solution of Sodium Citrate, C.P., in double-distilled water. It will be best to make not less than 500 cc. to insure accuracy.

(4). Hydrogen Peroxide, C.P., (10 volumes, 3%). This should be a freshly opened bottle. Never use one that has been standing uncorked at room temperature for any length of time.

### 3. Procedure.

(1). Pipette 10 cc. of the gold chloride solution into 950 cc. of double-distilled water in the 2000 cc. Erlenmeyer flask.

(2). Heat as rapidly as possible to between 90 and 95 degrees C. No higher and no lower.

(3). Remove thermometer from flask as soon as it records 92 degrees.

(4). Without removal from the flame, add 50 cc. of the citrate solution.

(5). As soon as the solution comes to a boil again add quickly 0.77 cc. of hydrogen peroxide (or ten drops from a standard pipette). When drawing up the peroxide into the pipette take care to exclude the many small bubbles that form in a freshly opened bottle.

(6). Remove from flame, cool and store in a dark place at room temperature. Cork tightly with a tinfoil covered stopper.

DO NOT SHAKE THE FLASK DURING PROCEDURE.

This method takes about ten minutes when a hot flame is used.

When the solution begins to reheat after the addition of the citrate, a slight bluish tinge begins to appear which gradually changes to the standard color during three minutes of boiling. No further change takes place thereafter. This was the original procedure but does not give as sensitive a product as is desirable. The addition of the peroxide, with its immediate color change, gives the required sensitivity. The solution must not be boiled after the peroxide is added. No saline, edestine or pH titration is necessary if the final color is right.

The standard color shows no trace of a bluish tinge. It may be best described as a brownish-yellow shade of Burgundy red.



## Colloidal Gold Procedure

1. Place 10 chemically clean test tubes in a rack.
2. In the first tube place 0.9 cc of a .4% salt solution and in each of the remaining 9 tubes place 0.5 cc amounts of salt solution.
3. To the first tube add 0.1 cc of the spinal fluid to be tested and mix thoroughly.
4. Remove 0.5 cc of the mixture from tube #1 and place in the second tube, thoroughly mixing as before.
5. Remove 0.5 cc from tube #2 and carry to the third; continue this transfer until the 10th tube is reached: from the latter discard 0.5 cc.
6. Add to each tube 2.5 cc of the gold chloride solution.
7. Mix thoroughly by rotation of the tubes, set aside at room temperature for 24 hours. Read results.





## The Two-Tube Kolmer Test. (Sheep system.)

### Glassware and Apparatus.

- Pipettes: 0.2 cc., graduated to 0.01 cc.
- 1.0 cc., graduated to 0.01 cc.
- 10.0 cc., graduated to 0.1 cc.
- Test tubes, 100 x 12 mm., heavy wall, without lip.
- Test tube racks, carrying two rows of ten tubes each.
- Centrifuge and centrifuge tubes.
- Water baths: Inactivating, set at 56 deg. C.
- Incubating, set at 37 deg. C.
- Refrigerator, running at 6 to 8 deg. C.

All glassware should be chemically clean and should be used dry or rinsed out with normal saline solution just before using. Never use any glassware containing the slightest degree of water.

### Patient's Serum.

See section under Venipuncture. The serum must be inactivated at 56 deg. C. in the water bath for 30 minutes just before using. If the serum to be tested has previously been inactivated, only a 10-minute inactivation is necessary just before running the test.

### Salt Solution.

This is an isotonic solution of sodium chloride. Add 0.85 grams of chemically pure sodium chloride (Merck's Blue Label) to 100 cc. of distilled water.

### Sheep Cell Suspension (Indicator Antigen).

Collect the blood by bleeding the sheep from the external jugular vein into 1 to 3 per cent sodium citrate solution. The blood may be received into a flask containing a handful of sterile glass beads and shaken well to defibrinate it. Either method prevents clotting. The former method is preferable. Filter a small amount of the blood through cotton into graduated centrifuge tube, allowing twice as much blood as will be required for the test to be run. Add 2 or 3 volumes of salt solution. Centrifuge at 10th speed for 10 minutes. Repeat this washing 5 times. On the last washing, centrifuge at the 10th speed for exactly 15 minutes. Do not vary the time or speed, so as to insure the same per cent suspension when the cells are finally diluted for use in the test. Read the volume of the cells in the centrifuge tube, carefully remove the supernatant fluid, and prepare a 2% suspension by washing the cells into a flask with 49 volumes of salt solution. Always shake well before using to secure an even suspension, as the cells rapidly settle to the bottom of the flask on standing.

### Complement.

See under section on Complement in directions for the Craig-Wassermann.

## Amboceptor.

Glycerolized amboceptor is prepared at the Army Medical School and may be obtained by requisition.

## Antigen.

While the titration factors are given on all reagents issued by the Army Medical School, it is advisable to keep watch that they retain their potency. Once in three weeks is about the proper frequency to check up by titration. The technique is as follows:

## Titration of Amboceptor.

1. Prepare a dilution of 1:100 amboceptor as follows:

Glycerolized amboceptor (50%)	2 cc.
Salt solution	94 cc.
Phenol (5% in salt solution)	4 cc.

This is to be kept in the refrigerator as a stock solution and is good for several weeks.

2. Dilute this stock solution for the titration as follows:

Stock amboceptor (1:100)	0.5 cc.
Salt solution	4.5 cc.

This will be 1:1000 in strength.

3. In a series of 10 tubes, prepare higher dilutions as follows:

#1.	Amboceptor 1:1000,	0.5 cc. plus no saline.	
#2.	"	" 0.5 cc. plus 0.5 cc. saline	(1:2000).
#3.	"	" 0.5 cc. plus 1.0 cc. "	(1:3000).
#4.	"	" 0.5 cc. plus 1.5 cc. "	(1:4000).
#5.	"	" 0.5 cc. plus 2.0 cc. "	(1:5000).
#6.	"	1:3000, 0.5 cc. plus 0.5 cc. "	(1:6000).
#7.	"	1:4000, 0.5 cc. plus 0.5 cc. "	(1:8000).
#8.	"	1:5000, 0.5 cc. plus 0.5 cc. "	(1:10000).
#9.	"	1:6000, 0.5 cc. plus 0.5 cc. "	(1:12000).
#10.	"	1:8000, 0.5 cc. plus 0.5 cc. "	(1:16000).

Mix the contents of each tube thoroughly.

4. Prepare a 1:30 dilution of the complement (see Complement under Craig-Jassermann) by diluting 0.2 cc. of the regenerated complement with 5.8 cc. of salt solution.

5. Prepare a 2% suspension of sheep cells in salt solution.

6. In a series of 10 tubes set up the amboceptor titration as shown in the following table:

Tube.	Amboceptor, 0.5 cc.	Complement, 1:30.	Saline.	Sheep cells.
1.	1:1000	0.3 cc.	1.7 cc.	0.5 cc.
2.	1:2000	to all	to all	to all
3.	1:3000	tubes.	tubes.	tubes.
4.	1:4000			
5.	1:5000			
6.	1:6000			
7.	1:8000			
8.	1:10000			
9.	1:12000			
10.	1:16000			



Mix the contents of each tube thoroughly.

7. Incubate in the water-bath at 38 deg. C. for 1 hour.
8. Read the amboceptor unit. The unit is the highest dilution of amboceptor that gives complete hemolysis.

Two units of amboceptor are used in the complement and antigen titrations and in the final test. Example: if the unit equals 0.5 cc. of the 1:6000 dilution, then two units will equal 0.5 cc. of the 1:3000 dilution. Dilute just enough of the stock amboceptor for the titrations and the number of tests to be run.

#### Titration of Complement.

1. Prepare a 1:30 dilution of the complement.  
(See paragraph 4 under amboceptor titration.)
2. Dilute the antigen as indicated by the dilution factor on the antigen bottle, by placing the required amount of salt solution in a small flask and adding the antigen drop by drop, shaking the flask continually until the antigen has all been added. Prepare enough for the complement titration and for the final test.
3. In a series of 10 tubes, set up the titration as follows:

Tube.	Complement (1:30) cc.	Antigen Dose. cc.	Salt Solution cc.		Amboceptor 2 units cc.	Sheep Cells 2% cc.	
1.	0.1	0.5	1.4	Water	0.5	0.5	Water
2.	0.15	0.5	1.4	Bath	0.5	0.5	Bath
3.	0.2	0.5	1.3	37°C.	0.5	0.5	37°C.
4.	0.25	0.5	1.3	for	0.5	0.5	for
5.	0.3	0.5	1.2	one	0.5	0.5	one
6.	0.35	0.5	1.2	hour.	0.5	0.5	hour.
7.	0.4	0.5	1.1		0.5	0.5	
8.	0.45	0.5	1.1		0.5	0.5	
9.	0.5	0.5	1.0		0.5	0.5	
10.	none	none	2.5		none	0.5	

The smallest amount of complement just giving sparkling hemolysis is the exact unit. The next higher tube is the full unit, which contains 0.05 cc. more complement. In the antigen titration and in the final test, two full units are used and are so diluted as to be contained in 1.0 cc. as in the following example:

Exact unit            0.3 cc.  
Full unit            0.35 cc.  
Dose (2 full units) 0.7 cc.

To calculate the dilution to employ so that 1.0 cc. will contain the dose of 2 full units, divide 30 by the dose (0.7). This equals 43, therefore 1.0 cc. of a 1:43 dilution will contain the required 2 full units.

## Titration of Antigen.

1. Prepare a 1:80 dilution of antigen by adding 0.1 cc., drop by drop, with continual shaking, to 7.9 cc. of salt solution in a large test tube or a small flask.
2. Higher dilutions are then prepared as follows:
  - 4 cc. of 1:80 plus 4 cc. of salt solution equals 1:160 dilution.
  - 4 cc. of 1:160 plus 4 cc. of salt solution equals 1:320 dilution.
  - 4 cc. of 1:320 plus 4 cc. of salt solution equals 1:640 dilution.
  - 4 cc. of 1:640 plus 4 cc. of salt solution equals 1:1280 dilution.
  - 4 cc. of 1:1280 plus 4 cc. of salt solution equals 1:2560 dilution.
3. Arrange 5 rows of test tubes with 6 tubes in each row.
4. In the first tube of each row place 0.5 cc. dilution 1:80.  
 In the 2nd tube of each row place 0.5 cc. dilution 1:160.  
 In the 3rd tube of each row place 0.5 cc. dilution 1:320.  
 In the 4th tube of each row place 0.5 cc. dilution 1:640.  
 In the 5th tube of each row place 0.5 cc. dilution 1:1280.  
 In the 6th tube of each row place 0.5 cc. dilution 1:2560.
5. Heat 3 cc. of moderately to strongly positive syphilitic serum in a water bath at 55 deg. C. for 15 to 20 minutes and prepare 5 dilutions in large test tubes as follows:

Tube.	Serum, cc.	Saline, cc.	Resulting Dilution.	cc. of Serum in 0.5 cc. of Dilution.
1.	1.0	4.0	1:5	0.1
2.	0.5	4.5	1:10	0.05
3.	0.5	9.5	1:20	0.025
4.	2.0(1:20)	2.0	1:40	0.0125
5.	1.0(1:20)	4.0	1:100	0.005

6. Add 0.5 of 1:5 dilution to each of the 6 tubes of the 1st row.  
 Add 0.5 of 1:10 " " " " " " " " 2nd "  
 " 0.5 " 1:20 " " " " " " " " 3rd "  
 " 0.5 " 1:40 " " " " " " " " 4th "  
 " 0.5 " 1:100 " " " " " " " " 5th "
7. Add 1.0 cc. of complement dilution carrying 2 full units to all 30 tubes.
8. In a separate rack, set up a serum control carrying 0.5 cc. of 1:5 serum and 1.0 cc. of complement (2 full units); also a hemolytic system control carrying 1.0 cc. of salt solution and 1.0 cc. of complement (2 full units).
9. Shake the tubes gently and place in the refrigerator at 6 to 8 deg. C. for 15 to 18 hours, followed by water bath at 37 deg. C. for 10 minutes.
10. Add 0.5 cc. of amboceptor (2 units) and 0.5 cc. of a 2% suspension of sheep cells to all 30 tubes and to the control tubes.



11. Mix thoroughly and place in water bath at 37 deg. C. for one hour and make readings. The serum and hemolytic controls should show complete hemolysis.

12. Chart the results as per the following example observed with a strongly positive serum:

Serum (in 0.5 cc.)	Antigen dilutions (in 0.5 cc. dose).					
	1:80	1:160	1:320	1:640	1:1280	1:2560
0.005	-	-	+++	-	-	-
0.0125	-	+	++++	++++	++	+
0.025	+	++++	++++	++++	++++	+
0.05	+++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	+++

The dose of antigen to employ in the final test is the largest amount giving a ++++ reaction with the smallest amount of serum. If three dilutions of antigen give ++++ reactions with the smallest amount of serum, the dose is midway between the highest and the lowest.

#### Procedure for the Test.

Having ascertained the exact amounts of the reagents to be used by the above methods, set up the two tube Kolmer test on the various blood sera for diagnosis as indicated in the following table:

Tube No.	Patient's Serum, cc.	Antigen, (10 units) cc.	Complement (2 full units) cc.	Amboceptor (2 units) cc.	Sheep Cells (2%) cc.
1.	0.2	0.5	1.0	0.5	0.5
2.	0.2	none (0.5 cc. saline)	1.0	0.5	0.5

Ten minutes at room temp.

Refrigerator at 6 to 8° C. 15 to 18 hours, then 10 to 15 minutes in water bath at 37° C.

Water bath at 37° C. for one hour. Then read.

Tube number 1 is the test tube; number 2 tube is the serum control tube and should show complete hemolysis. If tube 2 shows any residual cells, the serum is anticomplementary and should be so reported.

An antigen, an amboceptor and a sheep cell control should be set up with each lot of sera tested, as follows:

Tube No.	Saline cc.	Antigen cc.	Complement (2 units) cc.	Amboceptor (2 units) cc.	Sheep Cells (2%) cc.	
1.	0.5	0.5	1.0	0.5	0.5	Antigen control should show complete hemolysis
2.	1.0	None	1.0	0.5	0.5	Amboceptor control should show complete hemolysis
3.	2.5	None	None	None	0.5	Sheep Cell control should show no hemolysis

Wait ten minutes at room temp.

Incubate in refrigerator at 6 to 8°C. for 15 to 18 hours, then 10 to 15 minutes in water bath at 37°C.

Incubate in water bath at 37°C. for one hour.

A control, consisting of a known positive and a known negative serum should also accompany each lot of sera tested.

As in the Craig-Wassermann Test, the readings are made according to the amount of cells remaining in tube Number 1 for each serum tested. The readings are best made by plus signs, thus:

++++	=	no hemolysis)	) Reported as "Positive"
+++	=	25% "	
++	=	50% "	) Reported as "Doubtful"
+	=	75% "	
-	=	100% "	Reported as "Negative"

#### Spinal Fluids.

These are usually tested without any preliminary preparation as they do not contain enough complement to require inactivation by heating at 55°C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged.



The following table shows the set-up for complement fixation tests on spinal fluids for syphilis:

Tube No.	Spinal Fluids cc.	Antigen cc.		Complement (2 units)		Amboceptor (2 units) cc.	Sheep Cells (2%)	
1.	0.5	0.5	Wait ten minutes room temp.	1.0	Incubate in refrigerator at 6 to 8° C. for 15 to 18 hours, then 10 to 15 minutes in water bath at 37° C.	0.5	0.5	Incubate in water bath at 37° C. for one hour.
2.	0.5	none		1.0		0.5	0.5	

Tube number 2 is the control tube and should show complete hemolysis. The antigen, amboceptor and sheep cell control should be run with each lot, the same as for blood serum.

A control, consisting of a known positive and a known negative fluid should also accompany each lot of fluids tested.

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## BRIEF OUTLINES OF TECHNIQUE

In

1

## Histo-Pathology

## Chapter 1

## General Introduction

Common to all forms of life is the body substance called protoplasm. Protoplasm is a material which exists in a more or less gelatinous or semi-solid state. It is the "physical basis of life." Chemically protoplasm is a complex mixture of substances that vary from time to time. It contains proteins and their derivatives, lipoids and fatty bodies, carbohydrates, inorganic salts and water. In plant protoplasm the carbohydrates are the predominate substances, while proteins and lipoids are predominate in animal protoplasm. From the physical aspect, living protoplasm displays the properties of a complex colloidal system, and its behavior is usually that of a viscous liquid. The viscosity varies in degree, at the outer limits of the protoplasmic body it is greater; while inside, the contents exist in a more fluid state, apparently in the nature of a watery colloidal solution. In the watery colloidal phase of living protoplasm are suspended multitudes of ultramicroscopical particles or droplets, electrically charged, which in many instances are manifested by what is known as Brownian movement.

In order that the life phenomena may be sustained, and be enabled to carry out their purposes, protoplasm is organized into more or less complex units called cells (See Illustration Number 1). Basically, the general animal cell is composed of its body substance called cytoplasm and the nucleus which is situated within the cytoplasmic substance. The outside surfaces of the cell body is limited by the cell membrane. Within the membrane the cytoplasm is divided into two kinds, the portion of the cytoplasm situated immediately adjacent to the cell membrane is called exoplasm while that on the interior of the cell is the endoplasm. The nucleus of the cell is separated from the cell body or cytoplasm by a nuclear membrane. In many respects the nucleus is the vital part of the cell, and within it are situated many other important bodies.

Groups of specialized cells form tissues, tissues become associated to form organs, organs produce systems, and systems form the body.

The science that deals with tissues is called histology and it includes within its scope cytology the science that deals with cells. Briefly, the tissues of the animal body may be classified as follows:

1. Fluid tissues.

The fluid tissues comprise the blood and lymph.

2. Stationary tissues.

- (1) Epithelial tissues.

These are the most primitive of all tissues. They clothe outer surface of the body; line various cavities and passages, including the blood channels; secrete and excrete different substances; and give rise to the sex cells. They come in contact with other stationary tissues on one surface only.

- (2) Connective and Supporting tissues.

The connective and supporting tissues of the vertebrates are situated on the inside of the body. The cells of these tissues do not form layers, as epithelial tissues seem to do, but are massed together with irregularity. Their intercellular substances are usually much in evidence.

The mission of connective tissues is filling space between organs and groups of organs. There are five different sorts of connective tissue: (1) gelatinous; (2) notochordal; (3) reticular; (4) adipose; and (5) fibrillar.

The supporting tissues consist of cartilage and bone.

The tissues will be taken up more in detail in a later chapter.



Pathology may be defined as the study of the causes of disease, and includes the study of structural, chemical and functional alterations in the body resulting from disease. The study of the disturbances of function is called pathological physiology, and the study of structural changes is pathological anatomy and includes histo-pathology. Histo-pathology deals with the structural and chemical changes in cells and tissues. This is the subject which the histo-pathological technician is interested in. It would be the ultimate aim of the histo-pathological technician to move his specimen through such processes as would ultimately reveal a precise picture of the chemical and structural alterations which have taken place within the cells and tissues in his specimen. To enable such technician to attain this end only a few procedures, carefully executed, are necessary, namely, fixing, embedding, cutting, and staining.

## Chapter 2

### Fixing and Preserving

Comprising the methods for the microscopic study of animal cells and tissues are two general classes of procedures, namely, General procedures and Special procedures. General procedures, which are some times called normal procedures, consist in carefully fixing the structural elements to be examined, staining with a nuclear stain, counter staining if desirable, dehydrating with alcohol, removing the alcohol with some clearing agent, then mounting in balsam. It is within the scope of the general class of procedures that a piece of histological work is often planned and finished. Special procedures consist in the examination of living tissue elements in situ or in indifferent media; fixation with special fixing agents; staining with special stains; dissociation by teasing or maceration; and the like.

There is a further distinction which may be made in methods of procedure. These methods consist of Preliminary processes and Ulterior processes. Preliminary processes include that group of processes whose object it is to get the tissues into a fit state for passing unharmed through all the ulterior processes to which it may be desired to submit them. Preliminary processes comprehend the operations of killing; fixing; washing, and such other manipulations necessary for removing the fixing agents from the tissues, and substituting for them the preservative liquid or other reagents which it is desired to employ. Ulterior processes include such steps as dehydration; removal of the dehydrating agent; clearing; embedding; sectioning; treatment of sections; and mounting.

Fixing is the first preliminary process and it is composed of two phases, namely, killing and hardening. Killing is the first phase implied in the process of fixing, and by killing is meant a rapid dissipation of life in a tissue element, so that the element may not have time to change the form it maintained during life, but is sustained in death in the attitude it normally had during life. Hardening is the second phase of the process of fixing, and by hardening is meant a continuation of the process of treatment, which effected the rapid death of the tissue element, to such a degree as may enable the element to resist without further change of form the action of the reagents with which it may subsequently be treated. In almost all routine work one and the same reagent will suffice both for rapid killing of the tissue element and for hardening it.



In routine practice, a tissue element is submitted to the treatment of the fixing agent for a certain number of hours; then treated for a similar number of hours with some other agent, such as alcohol or tap water, to remove the fixing agent; and is then preserved in 80 per cent alcohol for further manipulation.

To facilitate the rapid penetration of the fixing agent, the tissues are originally divided, with a very sharp knife or scapel into the smallest portions that can conveniently be employed. Heat hastens the penetration of fixing agents. The fixing agent may be first warmed, the tissues placed into it and the whole set into an incubator with a temperature above that of the room. In some instances the tissues may be submitted to the action of a boiling fixing solution, but for routine purposes this should be avoided. It is practiced to some extent in connection with frozen section work where a diagnosis is desired within a few minutes. Cold appears to have little if any effect on the action of most fixing agents, and for this reason very cold fixing agents are nowadays used for some purposes.

Often it is quite difficult to make a choice of the proper fixing reagent for a given tissue. The kind of fixing agent to be used largely depends on the nature of the pathological lesions present or suspected and on the purpose for which the tissue is preserved. Fixing reagents are very numerous, but among the many there are a few outstanding ones. Some of the commonly used ones are outlined below.

#### 1. Alcohol.

Alcohol is a fair general fixing agent; it was formerly much in use, but its place is largely taken nowadays by formaldehyde. Such materials as bacteria, fibrin, mucus, various pigments, elastic fibers, and certain cytoplasmic granules stain well after being fixed in alcohol. It is an excellent preservative for glycogen in the cell and allows it to be stained differentially. Among its disadvantages, it tends to shrink tissues more or less, and does not give the tissues so good a consistence as some of the other fixatives. In the Army laboratories, the strengths of the alcohols ordinarily available are 95 per cent and absolute. Tissues submitted to either of these percentages shrink a great deal. The exposed surfaces of the pieces of tissue usually become extremely hard, and the outer layers of cells of the tissue often become shrunken and flattened almost as though they were dried in the air.

On the inside of this hard casing, where the alcohol has penetrated more slowly and has undergone dilution to some extent by the fluid of the tissues, the cells usually appear to be much better preserved.

Where alcohol is employed as a routine fixative, it is always advisable to place tissues at first into 80 per cent alcohol for two to four hours, and then replace the 80 per cent with 95 per cent or absolute alcohol. Specimens of tissues to be fixed should be cut into thin slices, 2 to 5 mm. thick, and kept in the upper part of the alcohol by means of absorbent cotton. The volume of alcohol used for fixing should be from fifteen to twenty times as great as the specimen. Specimens are fixed in alcohol usually from 6 to 24 hours. The alcohol should be changed at least once during fixing especially when 95 per cent is used. For preserving after fixing, specimens should be returned to 80 per cent alcohol.

## 2. Carnoy's Fluid.

This is an alcoholic preparation, and is one of the most penetrating and quickly acting fixatives known. It is much used by the French microscopists. Both nuclei and cytoplasm are well preserved. It has the following formula:

Alcohol, ethyl, absolute	60 parts
Chloroform	30 parts
Acid, acetic, glacial	10 parts

Tissues are fixed in this preparation for  $1\frac{1}{2}$  to 3 hours. Tissues fixed in Carnoy's fluid should be transferred to absolute alcohol and prepared and sectioned from there. This fixative should be freshly made up for each occasion. Specimens should never be washed in water after fixing in this agent.

## 3. Alcohol-Formaldehyde.

This is a very useful mixture, and is often employed for rapid diagnosis of routine surgical specimens. It has the following formula:

Formaldehyde, full strength	10 cc.
Alcohol, ethyl, 95 per cent	90 cc.



#### 4. Formaldehyde.

Formaldehyde penetrates very rapidly and is therefore applicable to large objects. This agent may be followed by most stains. Formaldehyde is commonly used in clinical work, when, because of its convenience, rapidity, and general applicability it lends itself to the conditions of routine work. It is a very useful agent for fixing and preserving gross specimens as it gives them a rather tough elastic consistence, and preserves the normal color usually better than most other routine fixing fluids. Generally considered, however, formaldehyde as a fixative for specimens that are to be embedded in paraffin is not recommended unless combined with other reagents, such as potassium bichromate in Orth's fluid, as it does not seem to harden the tissue elements sufficiently to enable them to resist the shrinking effects of alcohol and heat in prolonged exposures necessary in the process of embedding. Many workers do not recommend it for beginners in histology, but for most tissues fixed in formaldehyde, embedded in paraffin, cut with very sharp knives, and mordanted for 24 hours in Zenker's fluid, beautiful results by routine staining may be obtained by beginners.

Formaldehyde is an excellent fixing agent for frozen section work and is also very good for celloidin. It preserves myelin and is therefore the best fixative for the central nervous system. Formaldehyde dissolves glycogen, uric acid and sodium hippurate crystals; most frequently changes bile concretions from a yellow to a green color; and frequently gives rise in the tissues to a fine, dark brown or black crystalline precipitate derived from laked hemoglobin; it is not particularly a good preserver of iron and other pigments.

As a fixative, formaldehyde is usually used in what is called the 10 per cent solution. Formaldehyde is a gas which will go into solution in water to the extent of about 40 per cent, and the commercial product as obtained for routine laboratory purposes is a 40 per cent solution. In making up the routine fixing solution this factor is disregarded, and the fixing solution is made by using 10 parts of the 40 per cent solution and 90 parts of water and this gives what is generally known as the 10 per cent formaldehyde solution. Some workers use physiological saline instead of water to make the final solution.

Formaldehyde is a good preservative as well as a good fixative, but unfortunately on long standing formic acid gradually develops in it, rendering it acid and therefore exerting an injurious action on tissues preserved in it. To meet this situation, it is advisable to neutralize the prepared solution; neutralization is easily accomplished by adding calcium carbonate in excess to the 10 per cent solution. This will render the solution slightly alkaline, but for routine histo-pathological work it will not be of any disadvantage because most postmortem tissues are acid in reaction. Calcium carbonate will not neutralize full strength formaldehyde. For some procedures however, which will always be indicated in the outlines for the particular piece of work, it is desirable to employ an acidulated formaldehyde. In these cases, 5 per cent by volume of glacial acetic acid is added to the 10 per cent formaldehyde solution. This procedure is for particular cases and the tissues cannot be left in the mixture, but must be transferred after 24 hours to neutral formaldehyde.

On the market formaldehyde may have one of several names, such as Formol, Formalin, Formalose, and others, but when a product with one of these names is employed it is used in the same way as formaldehyde. Formaldehyde is obtained by the oxidation of methyl alcohol and in chemical literature it is sometimes called formic aldehyde or methyl aldehyde. If formaldehyde is kept in bottles in the light over long periods of time; that is, the full strength formaldehyde, it partially decomposes forming a white deposit of paraformaldehyde which settles to the bottom of the bottle. This process of decomposition can usually be avoided by the addition of a small amount of glycerin to the full strength formaldehyde. Tissues fixed in formaldehyde may remain in this solution as long as desired.



## 5. Zenker's Fluid.

### Formula: Zenker's Fluid.

Potassium bichromate	2-2.5 gm
Mercuric chloride (corrosive sublimate)	6 "
Water, distilled	100 cc
Acid, acetic, glacial	5 "

The potassium bichromate and the mercuric chloride are dissolved in water by the aid of heat. The glacial acetic acid is not added to the stock solution, but is added in the proper proportion to the amount to be used for fixing the tissues, immediately before the tissues are placed in the fluid.

After more than 40 years of constant trial, most histologists agree that Zenker's fluid is the most efficient general fixative yet available. It is universally applicable to almost all routine work in histo-pathology. However, in all important cases, tissues should be fixed both in Zenker's fluid and in formaldehyde; a portion in Zenker's fluid for general histological study, and for the preservation of nuclear figures, bacteria and fibrils of all kinds; and another portion in formaldehyde for the preservation of fat, myelin, amyloid and hemosiderin.

Tissues are fixed in Zenker's fluid for 12 to 24 hours, washed in running tap water for 12 to 24 hours to remove the Zenker's, washed over night in 80 per alcohol, and then preserved in fresh 80 per cent alcohol, preferably in the dark. Alcohol very slowly extracts chrome salts from the tissues, especially when exposed to the light.

For routine work, many histologists first fix tissues in 10 per cent formaldehyde, prepare and section, and the sections on slides are then placed in Zenker's fluid for 24 hours, then washed in running tap water for 24 hours before staining. Sections cut from Zenker's fixed tissue as well as those mordanted in Zenker's after fixing in formaldehyde, must be treated with an iodine solution, preferably Lugol's solution, to remove the mercuric chloride. Treat the sections for 10 to 20 minutes in the iodine solution, then treat with a 0.5 per cent solution of sodium thiosulfate in water, to remove the iodine, for about 5 minutes. Wash for about 5 minutes in distilled water to remove the sodium thiosulfate.

## 6. Orth's Fluid.

## Formula: Orth's Fluid.

Potassium bichromate	2-2.5	gm
Formaldehyde, full strength	10	cc
Water, distilled	100	"

Add the formaldehyde just before using.

Fix tissues from 3 hours to 3 to 4 days. In the longer periods of fixing, such as 3 to 4 days, the mixture usually becomes dark and a crystalline deposit begins to form, as a result of the interaction of the formaldehyde and the chrome salts, but this deposit is immaterial as fixation is completed by this time. Slices of tissue from 3 to 5 mm. in thickness, can readily be fixed in the incubator in 3 hours. But in routine work fixation is continued from 3 to 4 days, at room temperature.

Orth's fluid is a good fixative for mitotic figures, red blood cells, bone, and colloid material, as it gives a firm consistence to the tissues. But in general, the histological detail is not so well presented as after Zenker's fluid.

Wash tissues in running tap water for 24 hours before preserving in 80 per cent alcohol.

## 7. Muller's Fluid.

## Formula: Muller's Fluid.

Potassium bichromate	2-2.5	gm
Sodium sulfate	1	"
Water, distilled	100	cc

This fluid fixes tissues very slowly, evenly, and with little or no shrinkage. It is however, a poor nuclear fixative, and does not permit any great variety of stains.

Tissues are left in this fluid from 6 to 8 weeks. The fluid is changed daily during the first week; and once a week thereafter. Wash the fixed tissues in running tap water for at least 12 hours before preserving in 80 per cent alcohol.



## 8. Helly's Fluid.

## Formula: Helly's Fluid.

Potassium bichromate	2.5 gm
Mercuric chloride	5-8 "
Distilled water	100 cc
Formaldehyde, full strength	5-10 cc

Add the formaldehyde just before use.

Tissues are fixed in this fluid from 12 to 24 hours. They are then washed in running tap water from 12 to 24 hours and preserved in 80 per cent alcohol.

## 9. Mercuric Chloride (Corrosive Sublimato).

When mercuric chloride is used alone as a fixative, it is used as a saturated aqueous solution (about 6.9 per cent) made with the aid of heat. It is usually always advisable to add 5 per cent glacial acetic acid just before use. Thin slices of tissue, 2-5 mm. in thickness, are fixed in this agent from 6 to 24 hours. Do not wash in water, but soak in several changes of 70 per cent alcohol and then preserve in 80 per cent alcohol.

Tissues fixed in this solution stain quickly and brilliantly in nearly all staining solutions. It is the only fixative after which the Heidenhain-Biondi triple stain gives good results. Mercuric chloride, when used alone as a fixative, causes great shrinkage of the cells. After the tissues have been cut, and before staining, remove the mercury with iodine as in the case of Zenker's.

## 10. Giemsa's Corrosive Sublimato-Alcohol Fixative.

## Formula: Giemsa's Corrosive Sublimato-Alcohol Fixative.

Mercuric chloride, saturated solution	
in water	2 parts
Alcohol, absolute	1 "

This fixative is the one usually recommended for tissues that are to be stained by Giemsa's method. Fix the tissues for 48 hours, renewing the fixing solution after the first 24 hours. The tissues may remain as long as 3 months in this fluid without disadvantage. The mercury must be removed from the sections before they are stained; with iodine as usual.

## 11. Bouin's Fluid.

Formula: Bouin's Fluid.

Picric acid, saturated aqueous solution	75 cc
Acetic acid glacial	5 "
Formaldehyde, full strength	25 "

Tissues are fixed in this fluid for 18 hours. They are then washed in 50 per cent alcohol, and then in 70 for a number of changes or until the picric acid is practically all removed. They can then be preserved in 80 per cent alcohol. Masson fixes in this fluid up to 3 days, pours off the fixative and covers the tissues with water, but does not wash them in it, until ready for use. The tissues appear to keep indefinitely this way.

## 12. Regaud's Fluid.

Formula: Regaud's Fluid.

Potassium bichromate, 3 per cent aqueous solution	80 cc
Formaldehyde, full strength	20 "

Tissues are fixed in this fluid for 4 days, changing to fresh fluid every day. They are then chromatinized for 8 days longer in 3 per cent potassium bichromate. Wash in running tap water for 24 hours and preserve in 80 per cent alcohol.

This fluid is recommended for fixing tissues containing Rickettsiae, and for mitochondria. It must be made fresh each time for use as it does not keep.

## 13. Flemming's Solution.

Formula: Flemming's Solution.

Osmic acid, 2 per cent aqueous solution	4 parts
Chromic acid, 1 per cent aqueous solution	15 "
Acetic acid glacial	1 part

It is advisable to keep the osmic acid in a 2 per cent and the chromic acid in a 1 per cent solution. The fixing solution can be made fresh at the time it is needed.

Fix in the solution from 1 to 3 days. Wash in running tap water 6 to 24 hours and then preserve in 80 per cent alcohol.

This solution is very slow in penetrating, therefore thin sections of tissue should be used, about 2mm. in thickness.



## 14. Hermann's Solution.

Formula: Hermann's Solution.

Osmic acid, 2 per cent aqueous solution	4 parts
Platinic chloride, 1 per cent aqueous solution	15 "
Acetic acid, glacial	1 part

This is a modification of Flemming's Solution, and should be employed in the same manner.

## 15. Marchi's Fluid.

Formula: Marchi's Fluid.

Muller's Fluid (Fixative No. 7 above)	2 parts
Osmic acid, 1 per cent aqueous solution	1 part

Small pieces of tissue are fixed in this fluid from 5 to 8 days. Wash thoroughly in running tap water and preserve in 80 per cent alcohol.

It is applicable to degenerated nerve fibers.

## 16. Boiling.

This method of fixing is useful under some circumstances, especially in cases where it is desired to demonstrate albumin in renal diseases and in edema of the lungs. Small pieces of the tissues not over 5 mm. thick are dropped into boiling water for 30 seconds to 2 minutes; cool quickly in cold water and make frozen sections, or preserve in 80 per cent alcohol. Often 10 per cent, or even undiluted formaldehyde, is used instead of water.

## Chapter 3

### Embedding and Sectioning

#### A. Embedding

The processes of embedding are employed for a twofold end. Firstly, they enable the technician to surround a piece of tissue, too small or too delicate to be firmly held by the fingers, or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the complete body thus formed the included specimen of tissue may be cut into sufficiently thin slices without distortion. Secondly, the embedding mass fills out the natural cavities of the piece of tissue, so that their lining membranes and other structures contained in them may be duly cut in situ; further, not only will the supporting mass surround each individual organ or part of any organ that may be present in the interior of tissue specimen, but also to fill with it each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that the thin slices cut from the mass all the minutest details of structure will precisely retain their natural relations of position.

There are two principal methods of embedding, namely the paraffin method and the colloidin method; these are the ones commonly employed in histo-pathological work. In both these methods it is necessary to first remove the preservative from the tissue specimen to be embedded.

##### 1. Paraffin Embedding.

The first step in preparation for embedding a piece of tissue in paraffin is to cut the pieces of tissue into thin, square or rectangular pieces not over 2 to 3 mm. The next step is removing the preserving agent. For almost all general work, the following schedule is very applicable:

1. Wash specimen in 95 per cent alcohol,  
2 changes, 6 to 24 hours
2. Into absolute alcohol, 2 changes, 6 to 24 hours
3. Into alcohol-xylol, equal parts  
absolute alcohol and xylol, 6 to 24 hours
4. Into oil of cedarwood, clearing,  
2 changes, 6 to 24 hours
5. Xylol, to remove cedar oil,  
2 changes 30 minutes
6. Paraffin bath until no odor of oil  
is present, 2 to 8 hours
7. Block and cool quickly in cold water



## 2. Celloidin Embedding

The following schedule works very satisfactorily for celloidin embedding:

1. Place small pieces of tissue into 95 per cent alcohol, 2 changes, 24 hours
2. Into absolute alcohol, 2 changes, 24 hours
3. Into alcohol-ether, equal parts of absolute alcohol and ether, 24 hours
4. Celloidin, thin ) 24 hours
5. Celloidin, thick ) 1 or more weeks
6. Mount on blocks of vulcanized fiber.
7. Harden in chldroform for 1 to 2 hours.
8. Preserve in 80 per cent alcohol.

### B. Cutting Sections.

#### 1. Cutting Paraffin Sections.

After the block of paraffin has hardened it is removed from the embedding dish, and the specimens are cut apart with a scalpel and each block is fastened to a specimen disc by heating the latter in a flame until it will just melt the paraffin when the block is held in proper position against it. Now quickly dip the disc and specimen in cold water to cool.

The upper surface of the paraffin should now be shaved down to the specimen. The four sides are to be carefully trimmed; the upper and lower surfaces should be parallel and not cut too close to the specimen, otherwise the sections will not adhere to each other cutting ribbons; the lateral surfaces should as a rule be cut close to the tissue, especially if very thin sections are desired, because if a rim of paraffin is left it is likely to cause wrinkling of the section. The holder is now finally adjusted in the paraffin microtome, or placed away until needed.

Paraffin sections should not be cut over 7 microns in thickness, preferably thinner if possible. To get good sections that will adhere to each other and form a ribbon the temperature of the room must be regulated to suit the degree of hardness of the paraffin used. An open window will often make all the difference needed to obtain good results. The harder the paraffin the warmer the room must be. The temperature can be raised by burning a Bunsen flame near the microtome, or lowered by the presence of a lump of ice. It will often be found advantageous to dip the holder and paraffin block into ice water or to apply ice to the specimen, when fixed in the microtome, for a few minutes just before cutting.

The ribbons of sections as cut are laid on the surface of a dish or pan of warm water, about 44 degrees Centigrade, and if necessary gently stretched to remove all wrinkles.

When paraffin sections are cut in ribbons and spread onto water, as many sections as desired for each slide must be separated from the ribbon. This is easily accomplished by heating a common or dissecting needle in a flame and then passing between the sections in the ribbon; separating from one end so that the section for the slides can be moved away. After several sections have been separated, a drop of Mayer's albumin-glycerin mixture is placed on the slide and spread evenly with the tip of the finger or a towel until only a faint layer is left. Dip the slide into the water under the section or sections, arrange in order, then lift the slide and drain off the water. The slide is then placed in a slanting position until dry, when it is put into an open slide box and placed in the incubator for 12 hours or over night, at a temperature above 50 degrees Centigrade. This drying process attaches the specimens or sections firmly on the slide.

## 2. Cutting Celloidin Section.

After the celloidin mounts have been in 80 per cent alcohol from 1 to several hours, the celloidin is of the proper consistence for cutting. It is best to take a sharp knife and trim the top of the celloidin down to where the first good section of the specimen can be cut; this will save considerable wear on the microtome knife.

In cutting, the microtome knife should be fastened obliquely, so that as much of the edge of the knife as possible shall be used in making each section. The surface of the knife should be kept wet with 80 or 95 per cent alcohol, preferably from an overhanging dropping-bottle. Celloidin sections should be cut as thin as possible, from 10 to 15 microns for tissues in general. Sections of bone often have to be cut as thick as 20 to 22 microns in order to get sections of the whole block of tissue.

If sections curl, as often happens when they are thin, they are best flattened by unrolling them on the surface of the knife with a camel's hair brush just before the last edge of celloidin is cut through, as this serves to keep them in place during the process. This method can be used when the simple transferring of sections from alcohol to water is not sufficient to uncurl them.



Celloidin sections can be stained by nearly all methods, without the necessity of removing the celloidin. When necessary, however, the celloidin is readily removed by transferring the sections from absolute alcohol into oil of cloves or into a solution of equal parts of absolute alcohol and ether for 5 to 10 minutes, then passing them back through absolute into 95 per cent alcohol.

Celloidin sections should be placed onto the slide and attached as they are cut, although they can be stained and cleared before they are attached. They may be placed into 95 per cent alcohol as they are cut and left until all the sections desired are cut and then attached. From 95 per cent alcohol the section may be placed on the slide, pressed firmly with filter paper against the slide and then pour over it ether vapor from a bottle half full of ether. Follow slowly along the edges of the celloidin and the frills in it will soften down. Then dip the slide into 80 per cent alcohol to harden the celloidin.

### 3. Cutting Frozen Sections

Frozen sections often enable the pathologist to make a diagnosis in a few minutes. They may be made either of fixed or unfixed materials or tissues.

Make very thin sections of slices of tissue  $\frac{1}{8}$  to be frozen, not thicker than 5 mm. Place a little water under the tissue on the freezing box so as to aid in attaching it securely. Immediately after freezing the piece of tissue will usually be too hard to cut; wait a few seconds, and cut a section or two at short intervals until the tissue is found to have a consistence yielding satisfactory sections. At this instance a number of sections should be cut in quick succession. They are placed as cut in water. Usually the frozen section is stained before it placed on the slide, however they may be attached to the slide first. Float the section onto the slide and spread it out evenly. Then cover the section carefully by means of a dropping bottle with 95 per cent or absolute alcohol, which acts as a fixative so as to avoid wrinkling; after about 30 second or a minute drain off the alcohol and blot with fine filter paper. For frozen sections made from fixed tissues, the following method is very efficient: Coat the slide with a thick layer of Mayer's albumin-glycerin mixture and float the section onto it, spreading it out smoothly. Wipe away most of the fluid from around the section and press the section onto the slide with smooth filter or blotting paper. Immediately cover it with equal parts of aniline and oil of cloves, and then immediately rinse off the mixture with 95 per cent alcohol. After immersing in water to remove the alcohol, the section thus attached to the slide is ready for staining and mounting.

## Chapter 4

## Stains and Staining Methods

## A. General Introduction

There are several methods by which stains can be classified, but for routine purposes they are usually divided into two main classes, namely, basic stains and acid stains. In the basic stains, it is the base or cation, which consists of a complex organic compound, that gives the color; while in the acid stains, the anion gives rise to color. In the basic stains the color cation is in union with some simple acid, usually hydrochloric or sulphuric, but often acetic. In the acid stains, the color anion is combined with an inorganic base, usually sodium. These two phenomena are well illustrated in the constitution of the two fuchsin. Basic fuchsin is the hydrochloride of the base rosaniline, and the staining properties of this stain are due to rosaniline, whereas acid fuchsin is the sodium salt of a sulphonic acid, and the coloring constituent is present as an acid. A neutral stain may be evolved by union between a color base and a color acid. The basic stains are salts of weak bases, amino- or amino-derivatives, with strong acids. They are electrolytically dissociated as salts; their solutions contain colored cation and undissociated salt together with colorless anions. Basic stains undergo hydrolytic dissociation to a notable degree. The acid stains are salts of fairly strong sulphonic acids with strong bases. The acid stains are electrolytically dissociated in solution to a large extent. Their solutions contain colored anions, colorless cations, usually sodium, together with undissociated salts. They undergo hydrolytic dissociation to a negligible degree, if at all.

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It is readily/therefore that a solution of a stain is a highly complex system from a physico-chemical point of view. This phenomenon taken together with the fact that the protoplasmic elements to be stained are present as separate phases in the system of staining where they exist either as liquids or solids constitute a heterogeneous condition. A staining process is a very complex one, one in which chemical differences, colloidal states, and boundary surfaces are all involved. Many theories have been advanced to explain the nature of the process of staining but no general agreement has yet been reached that will give a satisfactory solution to the problem.



The nucleus of the animal cell is usually acid in reaction, and the cytoplasm is alkaline. The basic stains therefore react with nuclear elements to give the color effects, and the acid stains react with the cytoplasmic substances. This definition is general of course. And this phenomenon must serve for us to conclude that, because a tissue element takes up a basic stain, the element has the chemical nature of an acid; it may have, but substances other than acids will take up basic stains. The reverse situation holds true for the acid stains. For example, methylene blue is a basic stain, but it can be employed to stain nerves. On the other hand, the acidic stains hematoxylin and carmine when employed in combination with alum give nuclear color effects. Usually a reaction of a stain varies according to the conditions present in the particular staining process.

#### B. Some Important Routine Stains.

##### (1) Hematoxylin.

This stain is one of the most important and valuable stains available. It is an organic product, being derived from logwood by extracting the wood with water in the presence of ether. It can be prepared in the laboratory by taking the logwood extract found in commerce and treating it with ether. The hematoxylin as found on the market is in the form of small crystals, sometimes acicular. Occasionally some samples found on the market are very white, this condition having been produced by using sulphur dioxide in the process of manufacture. A product of this character is unsatisfactory, the keeping qualities in solution have been injuriously affected. The color commonly found, of the desirable product, varies from light yellow to a rusty purple. When kept in the dry state, hematoxylin retains its staining properties indefinitely. Hematoxylin is soluble in water, glycerin, and alcohol.

The staining effects of hematoxylin is not due to hematoxylin itself, but is due to hematin which is formed as a product of oxidation. Hematin is a color acid, and must be formed before the hematoxylin solution can give its color effects. Hematin, as a finished product, can be obtained commercially, and the staining solution made directly from it. But this procedure is not always indicated for quite often the solution easily overoxidizes either in the bottle or on coming in contact with the tissue elements. It is always advisable to start from hematoxylin and develop the hematin by oxidation. Hematin, the staining factor of hematoxylin, is a very weak plasma stain when employed in the absence of a mordant, but when used with appropriate mordants it can be made to give a powerful nuclear stain as well as a selective plasma stain.

Below is a brief list of the various tissue elements and pathological products which hematoxylin in combination with certain metals can stain:

Tissue Element or Pathological Product	Metal
Nuclei	Aluminum, iron, tungsten
Myelin sheaths	Chromium, copper, iron
Elastic fibers	Iron
Collagen	Molybdenum
Fibroglia, myoglia, neuroglia and epithelial fibrils	Tungsten
Axis cylinders	Lead
Mucin	Iron
Fibrin	Tungsten



Formulae for the preparation of hematoxylin staining solutions are numerous. Below are listed a few of the important ones.

(1) Aqueous Alum Hematoxylin:

Hematoxylin	1 gm
Ammonium or potassium alum	20 gm
Water, distilled	400 cc
Thymol	1 gm

Dissolve the hematoxylin crystals in 100 cc of the distilled water by the aid of heat. Then dissolve the alum in the rest of the water, and add the two solutions together. Add the thymol; this prevents the growth of mold. Place the solution in a bottle or flask, lightly stopper with a plug of cotton and expose to the air and light for about 10 days to ripen, that is, for the formation of hematin through oxidation. The solution can be ripened immediately by adding 17.7 cc of a 1 per cent aqueous solution of potassium permanganate. After the ripening process has been completed, stopper the bottle tightly. The solution will keep at its best for 2 or 3 months.

As alum hematoxylin stains become older they stain more quickly, but often the staining is more diffusely. This condition can be readily corrected by adding 5 per cent solution of alum water. The stain becomes precise again. It is advisable to filter the portion of the stain to be used just before it is employed.

(2) Delafield's Alum Hematoxylin:

Hematoxylin	4 gm
Alcohol, ethyl, 95 per cent	25 cc
Ammonium alum, saturated	
aqueous solution (about	
15 per cent)	400 cc

Prepare the alum solution, dissolve the hematoxylin in the alcohol and add to the alum solution. Place the preparation into a bottle, fasten plain gauze over the top of the bottle and set in the light for about 4 days. Then filter the solution and add the following:

Glycerin	100 cc
Alcohol, ethyl, 95 per cent	100 cc

Allow the solution to stand in the light in the gauze covered bottle for about two weeks, then stopper tightly and set aside, preferably in a light place for about two months before using.

Delafield's hematoxylin may be ripened immediately by adding 7 per cent by volume of hydrogen peroxide. This solution will keep for years. This staining solution is more customarily used by filtering a few drops of the stain into tap water in a coplin jar, and stain sections in this preparation for several hours or over night.

### (3) Harris's Alum Hematoxylin:

Hematoxylin	1 gm
Alcohol, ethyl, absolute	10 cc
Ammonium or potassium alum	20 gm
Water, distilled	200 cc
Mercuric oxide	0.5 gm

Dissolve the alum in the water by the aid of heat, and ~~ix~~ dissolve the hematoxylin in the alcohol, then mix the two solutions together. Bring the mixture to a boil as rapidly as possible and then add the mercuric oxide. The solution immediately assumes a dark purple color. As soon as this occurs remove the preparation from the flame and cool by plunging into a basin of cold water. After the solution has cooled it is ready for use. This solution will keep for a long time in a tightly stoppered bottle. Some workers add 4 per cent by volume of glacial acetic acid which tends to increase the precision of the nuclear staining properties.

### (4) Ehrlich's Acid Alum Hematoxylin:

Hematoxylin	2 gm
Alcohol, ethyl, 95 per cent	100 cc
Water, distilled	100 cc
Glycerin	100 cc
Ammonium or potassium alum	3 gm
Glacial acetic acid	10 cc

The hematoxylin is dissolved in the alcohol, and the other ingredients are then added. Place a plug of cotton in the bottle and set aside for two weeks to ripen. The solution may be ripened at once by adding 0.4 gm sodium iodate. After ripening stopper the bottle tightly. This solution will keep for a long time.

This staining solution gives sharp nuclear colors but sections must be washed thoroughly in tap water to remove all traces of the acetic acid, and to produce a clear blue color.



(5) Mayer's Acid Alum Hematoxylin:

Hematoxylin	1 gm
Water, distilled	1000 cc
Sodium iodate	0.2 gm
Ammonium or potassium alum	50 gm
Citric acid	1 gm
Chloral hydrate	50 gm

The hematoxylin is dissolved in the water, using gentle heat if necessary. Then add the sodium iodate and the alum. Shake occasionally until the alum is dissolved, then add the citric acid and the chloral hydrate. The solution is ready for use immediately after it is made up. It will keep a long time.

The alum hematoxylin is used chiefly as a nuclear stain, alone, or followed by a counter stain. The principle of the staining method with these various solutions are about the same, but the time required depends upon the ripeness of the solution and on the fixative to which the tissues were submitted. For sections of tissue fixed in alcohol or formaldehyde, the time is usually a few minutes, more frequently 1 to 5 minutes, while for Zenker fixed material it is usually much longer, often 1 hour or more. Thorough washing in tap water or slightly alkalized water is always advisable to bring out a precise blue color of the nucleus.

(6) Mallory's Phosphotungstic Acid Hematoxylin:

Hematoxylin	1 gm
Phosphotungstic Acid	20 gm
Water, distilled	1000 cc

Dissolve the solid ingredients in separate portions of the water; the hematoxylin with the acid of heat. When cool, combine. No preservative is necessary. Spontaneous ripening requires several weeks, but it can be accomplished at once by adding 0.177 gm. of potassium permanganate.

## (7) Weigert's Iron Hematoxylin:

This preparation consists of two solutions, Solution A and Solution B each of which is prepared as follows:

## Solution A

Hematoxylin	1 gm
Alcohol, ethyl, 95 per cent	100 cc

## Solution B

Iron chloride (29 per cent aqueous solution)	4 cc
Water, distilled	95 cc
Hydrochloric acid	1 cc

For use, mix equal parts of Solution A and Solution B. The mixture assumes a dark black color. The two solutions should be added together just before use. This staining preparation will follow any fixative.

## (8) Heidenhain's Iron Hematoxylin:

This staining preparation consists of two separate solutions as follows:

## Solution A

Ammonio-ferric alum	2.5-4 gm
Water, distilled	100 cc

## Solution B

Hematoxylin	0.5-1 gm
Alcohol, ethyl, 95 per cent	10 cc
Water, distilled	100 cc

In selecting the iron alum select only the bright purple crystals. The iron solution should be a brilliant port wine color when filtered. Samples of the iron alum in the powder form or of a yellowish color should be discarded.



In preparing the hematoxylin solution, the hematoxylin is dissolved in the alcohol and then added to the water. Place the solution in a bottle, stopper with a cotton plug and allow the solution to ripen 4 or 5 weeks. For use, dilute the hematoxylin solution with equal parts of distilled water. This staining preparation will follow satisfactorily fixation in mercuric chloride, Zenker's fluid, alcohol and some other fixatives.

To employ the stain, mordant the sections in the ammonio-ferric alum solution for 3 to 12 hours. In connection with this, usually the iron alum solution is originally prepared in a 4 per cent solution and the mordanting is carried out in the full strength. As soon as the process of mordanting is completed, wash the sections very quickly in tap water. Where the 4 per cent iron alum solution has been employed it is necessary to wash very thoroughly in tap water, preferably in running tap water for several minutes. Then stain from 1 to 36 hours in the diluted hematoxylin. Wash the sections well in tap water. Differentiate the sections in the iron alum solution of about 2 per cent strength. The process of discoloration should be controlled under the microscope, being careful to wash the sections before each microscopic examination in tap water, which immediately halts the discolorization. After the sections have been sufficiently destained, they are washed in running tap water for at least 1 hour, then counter stained if desirable, passed through the alcohols, xylene, and mounted in balsam.

## 2. Carmine.

Carmine is derived from cochineal which is extracted from the bodies of certain female insects (*Dactylopius coccus*). The staining principle of carmine is carmic acid. Carmic acid can be obtained commercially in pure condition. The carmine stains are not generally employed in histo-pathological work, though in some processes they are indicated. Carmine solutions are usually used for one of four purposes, namely, (1) as a stain for nuclei in bulk, that is, before the specimen of tissue is sectioned (more useful to the embryologist), (2) to stain nuclei red in contrast with blue stains for iron, (3) to stain glycogen, and (4) as a stain for mucin. Generally the carmine solutions give good nuclear stains, but for the finer details in a specimen they are less precise in their effects than the alum hematoxylin. Below are listed some of the carmine solutions:

### (1) Grenacher's Alum Carmine:

Carmine powder	2 gm
Ammonium alum	3-5 gm
Water, distilled	100 cc
Thymol	1 gm

Combine the water, carmine, and alum and boil steadily for 1 hour. Add distilled water sufficient to make up for that lost by evaporation in boiling. Let cool, filter, and add the thymol. This solution is useful for both sections and tissues in bulk.

### (2) Grenacher's Alcoholic Borax Carmine:

Carmine powder	2-3 gm
Borax (Sodium borate)	4 gm
Water, distilled	100 cc
Alcohol, ethyl, 70 per cent	100 cc

The carmine and borax are ground thoroughly in a mortar, the water is added, and then the solution is brought to a boil and boiled until the substances are dissolved. After the mixture has cooled add the alcohol. Shake occasionally during a period of 3 or 4 weeks, then decant and filter. This solution is very useful for staining in bulk. Whole specimens of tissues are fixed, washed, and placed into 80 per cent alcohol from where they are transferred to the alcoholic borax carmine for about 3 days, depending on the size of the specimen; for small specimens 1 day will suffice. The tissue is then placed into 70 per cent alcohol containing 0.25 to 0.5 per cent hydrochloric acid where they are kept for 1 to 3 days, or until no more clouds of color are given off. Then wash out in 70 or 80 per cent alcohol for 1 to 3 days, changing once or twice. The tissues are now ready for the embedding process.



## (3) Orth's Lithium Carmine:

Carmine powder	2.5-5 gm
Lithium carbonate, saturated aqueous solution (about 1.25 per cent)	100 cc
Thymol	1 gm

The carmine powder is dissolved in the lithium carbonate solution and boiled for 10 to 15 minutes. When cool add the thymol. This preparation is often employed as a counter stain for bacteria in the Gram-Weigert method and when so used, it should be carefully filtered. This solution follows best after fixing in formaldehyde or alcohol.

## (4) Best's Carmine Stain for Glycogen:

Carmine powder	2 gm
Potassium carbonate	1 gm
Potassium chloride	5 gm
Water, distilled	60 cc

Mix the ingredients and boil gently and cautiously for several minutes, 5 to 10. When cool add:

Ammonia water, 26 to 28 per cent      20 cc.

This solution should be tightly stoppered and kept in the ice box as it deteriorates rapidly if left at room temperature

### 3. Artificial Stains.

Hematoxylin and carmine are natural dyes. Most of the other dyes generally used by the histologists are known as artificial dyes. They are also called aniline dyes. These stains are indispensable for certain purposes. Below is a list of tissue elements and pathological products which can be stained more or less specifically by these dyes, and the dyes capable of doing so:

Bacteria	methylene blue, crystal violet, basic fuchsin, and others.
Nuclei of animal cells	methylene blue, basic fuchsin, safranin, thionin, and others.
Fibrils and fibroglia	acid fuchsin, phloxine, eosin,
Collagen	aniline blue, acid fuchsin.
Elastic fibrils	basic fuchsin.
Nerve fibrils	methylene blue.
Amyloid	crystal violet, congo red
Fibrin	crystal violet.
Mucin	methylene blue, and others.

#### (1) Methylene Blue.

Methylene blue is an excellent nuclear stain for animal tissues and is also a very important bacterial stain. It is an excellent stain for nerve fibers, and stains mucin well.

It is always advisable to keep on hand a saturated solution in 95 per cent alcohol as a stock solution from which various strengths can be made.

#### (a) Loeffler's Methylene Blue Solution:

Methylene blue saturated (about 1.48 per cent) in 95 per cent ethyl alcohol	30 cc
Sodium hydroxide 1:10,000 aqueous solution	100 cc

This is one of the most important staining solutions of the aniline dyes in use. The preparation will keep for a long time.



## (b) Gabbet's Methylene Blue Solution:

Methylene blue powder	2 gm
Sulphuric acid	25 cc
Water, distilled	75 cc

This preparation is employed as a decolorizer and as a contrast stain for tubercle bacilli.

## (c) Unna's Alkaline Methylene Blue Solution:

Methylene blue powder	1 gm
Potassium carbonate	1 gm
Water, distilled	100 cc

This strongly alkaline preparation is exceedingly valuable as a general histological stain when used in combination with phloxine or eosin. The counter stain, that is, the eosin or phloxine should be applied before the methylene blue stain. The methylene preparation gives better results after it has been allowed to ripen for about two weeks.

## (2) Basic Fuchsin.

This is a very important stain. It is a basic stain and is quite often employed for the study of nuclear structure. Basic fuchsin is one of the important bacterial stains. It is best to keep on hand a saturated alcoholic solution (about 5.95 per cent) in 95 per cent alcohol.

## (a) Ziehl-Neelsen's Carbol Fuchsin:

Basic Fuchsin, saturated alcoholic solution	10 cc
Phenol water, 5 per cent	90 cc

The phenol water is prepared by shaking together 5 cc of melted phenol crystals and 95 cc of distilled water.

The staining solution should be filtered. It is a very powerful stain, stains quickly, keeps well, and can be employed for a variety of purposes. Its chief use is for staining tubercle bacilli.

## (b) Verhoeff's Carbol Fuchsin:

Basic fuchsin	2 gm
Alcohol, ethyl, absolute	50 cc
Phenol, melted crystals	25 cc

Combine the ingredients and place in the incubator over night to ensure complete solution. Cool and filter. This solution is very stable. It is useful for staining tubercle bacilli in sections.

## (c) Goodpasture's Stain:

Basic fuchsin	0.59 gm
Aniline	1 cc
Phenol crystals	1 gm
Alcohol ethyl, 30 per cent	100 cc

## (3) Crystal Violet.

This stain, because it is a definite chemical compound, is now generally substituted for both methyl violet and gentian violet in all histological methods. In all the older formulae where gentian violet is designated, crystal violet should be substituted.

## (a) Stirling's Gentian Violet:

Crystal violet	5 gm
Alcohol, ethyl, absolute	10 cc
Aniline	2 cc
Water, distilled	88 cc

This solution keeps remarkably well.

## (4) Eosin.

This is one of the important counter stains used in connections with the hematoxylin. It is an excellent cytoplasmic stain.



### C. Some Routine Staining Methods.

The staining methods outlined below are general in nature, being for teaching purposes only, and do not cover the entire field as would be found in one of the larger histo-pathological laboratories. They are intended for classes in pathological anatomy, and in no sense should it be concluded that they will meet all the demands of a general histo-pathological laboratory. They are intended to serve to acquaint the student with staining technique for histological methods in general.

Foremost among the nuclear stains is hematoxylin the preparations of which are divided into two classes for general purposes, namely, the alum hematoxylin and the iron hematoxylin. For general purposes, one method works very satisfactorily for all the alum hematoxylin; that is, the general principle is the same. Slightly modified methods are indicated for the iron hematoxylin.

#### 1. Method for Alum Hematoxylin.

- (1) For paraffin sections from tissues fixed in formaldehyde, alcohol, and most solutions not containing mercuric chloride:

Deparaffinize sections by passing through two washings of xylene, from 5 to 10 minutes for each xylene. Pass down through the graded alcohols to distilled water. Transfer to the hematoxylin solution for 1 to 5 minutes. Dip the slide once or twice into acid alcohol, and then wash thoroughly in tap water. Wash in tap water until the section assumes a bluish purple color. Rinse in distilled water, and pass up through the alcohols to 95 per cent; counter stain with eosin about 0.25 per cent dissolved in 95 per cent alcohol. Wash off excess eosin in 95 per cent alcohol, and pass into absolute alcohol, two changes, about 5 to 10 minutes in each. Clear in xylene, two washings, for 5 to 10 minutes each. Mount in balsam.

- (2) For paraffin sections cut from Zenker fixed, or in any other fixative which contained mercuric chloride:

Pass down through the alcohols to distilled water. Transfer to iodized alcohol (70 per cent alcohol in which iodine has been added till the alcohol assumes a port wine color) to remove the mercury, for about five minutes. Then pass into 0.5 per cent sodium thiosulphate, for five minutes, to remove the iodine. Wash in distilled water to remove the thiosulphate. Then the procedure is as above indicated.

### (3) For zenkerized sections (paraffin):

Quite often the histologist prefers to fix the tissues in formaldehyde, embed in paraffin and section, and then apply the Zenker solution.

For this method, the paraffin is removed from the sections; sections are passed down through the alcohols into distilled water. Place the sections in Zenker's fluid for 24 hours, wash in tap water for 24 hours, and apply the proper treatment to remove the mercury etc. The procedure is then as for (1) above.

## 2. Methods for the Iron Hematoxylin.

### (1) Weigert's Iron Hematoxylin: Any fixative may be used.

Remove the paraffin and pass the sections down to 40 per cent alcohol. Transfer to equal parts of Solution A and Solution B of Weigert and let stand for 10 or more minutes. Wash thoroughly in water. Counter stain if desirable. Dehydrate in the alcohols, clear in xylene and mount in balsam.

### (2) Hoidenhain's Iron Hematoxylin: Fixative: Mercuric chloride, Zenker's Fluid, Alcohol, or others.

The sections are deparaffinized, passed down through the alcohols to distilled water. Mordant in the iron alum solution for 3 to 12 hours. Wash quickly in tap water. Differentiate in the iron alum solution controlling the results with the microscope. Rinse the section in tap water, which immediately halts the decolorization, before each examination. Wash in running tap water for 1 hour. Counter stain if desirable. Dehydrate, clear and mount.

## 3. Methods for Staining Bacteria in Sections.

### (1) MacCallum's Modification of Goodpasture's Method for Gram-Positive and Gram-Negative Bacteria: Fixative: Zenker's preferable.

Stain sections, which have been deparaffinized, for 10 to 30 minutes in Goodpasture's stain (See under basic fuchsin). Wash in distilled water. Differentiate in strong formaldehyde until the bright red color changes to a clear rose. Wash in distilled water. Counterstain for 3 to 5 minutes in a saturated aqueous picric acid solution, or until the sections assume a purplish yellow color. Wash in water. Differentiate in 95 per cent alcohol which causes the red color to reappear. Wash in water, distilled.



Stain for 5 minutes in Stirling's crystal violet solution( See under crystal violet. Wash in distilled water. Immerse in Gram's iodine solution for 1 minute. Blot dry without washing in water. Treat sections with equal parts of aniline and xylene until no more color comes off. Rinse in two changes of xylene and mount in balsam.

Results: Gram-positive organism blue; Gram-negative organisms red; all other tissue elements various shades of red to purple.





## CLASSIFICATION OF HELMINTHS

The helminths of importance to the medical soldier may be classified in three main groups: Flukes (trematodes), tapeworms (cestodes), and roundworms (nematodes).

Adult flukes and tapeworms are usually flat, and are grouped, along with some non-parasitic forms, in the phylum Platyhelminthes (Platy= flat helminth= worm). Adult flukes resemble leeches in appearance although they are in no way related to this group. On the other hand adult tapeworms are somewhat ribbon-like, with a long series of segments set behind a very small head.

The roundworms (also called threadworms) are grouped in the phylum Nematelminthes (nema=thread helminth). Many of the smaller forms resemble bits of thread, but in the larger forms the worm-like character is more evident. These larger roundworms are the ones to which reference is usually made when a child, a puppy or a kitten is said to be "passing worms".

The life-cycles of many parasitic helminths are very complicated since they may involve several hosts. Some of the immature stages are recognizable only to experts. Lack of space prohibits the mention of more than a single life-cycle from each of the above groups. For details of these life-cycles, and those of other parasitic forms, the medical soldier is referred to standard works \* in this field.

**Life Cycle in a Fluke, the large Intestinal Fluke (*Fasciolopsis buski*).** Adult flukes living in the small intestine lay eggs which are passed out in the feces of the host. The larva (called a miracidium) develops in the egg, breaks out, swims around in the water, finds a snail, penetrates the soft parts, migrates through the snail's tissue and undergoes changes in structure. The larva is now called a sporocyst. Many smaller individuals (rediae) develop in each sporocyst, increase in size and rupture the sporocyst (still within the snail, however). Another set of rediae then develop in each redia (sing. of rediae). They may be considered as daughter rediae. Other larvae (cercariae) develop within the daughter rediae, many cercariae developing in each daughter redia. The cercariae escape from the daughter rediae, erupt from the snail's tissue and swim to plants on which the snails feed. Cercariae encyst on these plants and remain there until ingested by a suitable host. They are called metacercariae after encystment. In the intestinal tract of the host, the immature fluke escapes from the cyst and attaches itself to the wall of the intestine where it develops to the adult stage.

**Life-Cycle of a Tapeworm, the Beef Tapeworm (*Taenia saginata*).** The adult tapeworm in the small intestine either extrudes ripe eggs, or the terminal segments containing ripe eggs break off from the worm. In either event eggs are evacuated in the feces of the host. Eggs ingested by oxen hatch in the intestinal tract. The embryos burrow through the intestinal wall into the lymphatics or the blood stream. They are carried to various parts of the host's body and are filtered out in tissues, usually muscles, where the larvae develop



into mature cysts (cysticerci). When insufficiently cooked beef containing the cysticerci is eaten by man, the immature worms are set free and attach themselves to the wall of the small intestine where they develop into adults.

Life-Cycle of a Roundworm, the American Hookworm (*Necator americanus*). Adult females in the small intestine extrude eggs which are evacuated in the feces of the host. These eggs embryonate and hatch; the larvae (rhabditoid larvae) feed, and in a few days moult; then resume feeding and growing. These larvae then transform into another type (filariform larvae), moult, but remain within the "sheaths". After a quiescent period, the filariform larvae shed these "sheaths", and become active. This is the infective stage for man. On contact with human skin they penetrate it and enter the blood stream, whence they are carried through the heart to the lungs where they "break out" into the alveoli. They are then transported up the air passages to the epiglottis, then down the alimentary tract to the small intestine where they develop into adults. During this passage another moult occurs either in the trachea or in the small intestine. Still another moult takes place in the intestine.

#### GENERAL CONSIDERATIONS FOR LABORATORY DIAGNOSIS.

It may be seen from these life-cycles that worms living in various parts of the body may cause pathological changes with the appearance of certain clinical symptoms. Many cases of helminthic infection may be diagnosed by clinical symptoms alone, but it is always more reliable if the parasite itself (in any stage - egg, larva, or adult), or a part of the parasite, can be demonstrated. In many cases identity of the adult helminths is difficult to determine, and some of the larval forms are even more difficult. The eggs of the various species, however, are usually sufficiently distinctive, that, when eggs can be recovered, they serve as an excellent means of diagnosis in the routine laboratory.

Where diagnostic material (i.e., eggs, larvae, adults, or pieces of parasite) will be found, depends upon the life-cycle of the parasite. The adult large intestinal flukes live in the small intestine, and discharge their eggs directly into the lumen. Therefore, an examination of the host's feces for eggs, will reveal the presence or absence of these flukes. Beef tapeworms usually do not extrude eggs; the terminal segments of the worm, which contain ripe eggs, break off from the worm, and are passed out in the feces. Here diagnosis is based on recovery of part of the adult. Again fecal examination is useful in the case of hookworms, whose eggs are extruded directly into the lumen of the small intestine. It will be seen that the presence of a large number of forms can be diagnosed by an examination of the feces, even though some of the adults may not inhabit the intestine. However, since the life-cycles of the various helminths differ, the diagnostic material of some species may be found in other media. In the case of some of the filarial worms (nematodes) the eggs and larvae may be found in the blood where they have been deposited by the adult females. In trichinosis the larvae may be found in the muscle tissue, recovered by biopsying small bits of muscle. The presence of other forms may be revealed by an examination of the urine, or the sputum. Once again, the life-cycle of the worm determines where diagnostic material is most likely to be found; the clinical examination furnishes clues as to the offending parasite; and the laboratory examination confirms diagnosis.



The principal helminths causing disease in man are grouped below according to the medium in which diagnostic material is usually found. Species marked with an asterisk (\*) are common parasites of man.

Feces. \*Manson's Blood Fluke (Schistosomo mansoni) (Fig. ,R). Infection is acquired by swimming or wading in water containing the infective larval stage. These larvae (cercariae) penetrate the skin and enter the blood stream. The adults usually inhabit the mesenteric vessels draining the large bowel. Diagnosis is based on recovery of eggs in the feces of the host. Distributed in parts of Africa, particularly the Nile delta and a very large equatorial section extending East and West to both coasts. Also distributed in the northeastern section of South America, several of the Lesser Antilles, and Puerto Rico.

\*Oriental Blood Fluke (Schistosoma japonicum) (Fig. ,Q). Infection is acquired in a similar manner to Manson's Blood Fluke. Adults of this species also inhabit the mesenteric vessels draining the large bowel, and diagnosis is again based on recovery of eggs in the host's feces. Distribution is limited to the Orient.

Sheep Liver Fluke (Fasciola hepatica) (Fig. ,T). This parasite is rarely found in man. Infection is acquired by accidental ingestion of encysted larval forms (metacercariae). Adults live in the bile passages of the liver. Diagnosis is based on recovery of eggs in the feces of the host. World-wide in distribution.

\*Large Intestinal Fluke (Fasciolopsis buski) (Fig. ,T). Infection is acquired by accidental ingestion of infective larval cysts (metacercariae) usually while eating pods, stems, roots or bulbs of water plants, or in peeling water chestnuts with the teeth. The adults inhabit the duodenal region of the small intestine, and diagnosis is based on recovery of eggs in the host's feces. The eggs of this species cannot be differentiated from those of the sheep liver fluke in routine laboratory diagnosis. Distributed in the Orient.

\*Chinese Liver Fluke (Clonorchis sinensis) (Fig. ,E). Infection is acquired by ingestion of encysted larvae (metacercariae) during consumption of infected fresh-water fish. Adults live in the bile passage of the liver. The eggs carried into the intestine by the bile flow and evacuated in the host's feces, constitute the basis for diagnosis. Distributed in Sino-Japanese areas of the Orient.

\*Broad Fish Tapeworm (Diphyllobothrium latum) (Fig. ,C). This tapeworm is acquired by eating raw, salted, pickled, or insufficiently cooked fresh-water fish infected with the larval form (sparganum). The adults inhabit the small intestine where they lay eggs. Diagnosis is based on recovery of the eggs in the host's feces. The infection is common in the North and Central regions of Europe and in the Great Lakes Region of the United States.

\*Beef Tapeworm (Taenia saginata) (Fig. ,L and N). This is the most common human tapeworm. It is acquired by eating insufficiently cooked beef that contains the larval form (cysticercus). The adults usually inhabit the small intestine, and specific diagnosis is based on recovering segments of the adult worms



in the host's feces. Eggs may also be recovered from the feces, but are not sufficiently distinguishable from those of the pork tapeworm for practical diagnosis. Since segments of the pork tapeworm may also be recovered from the feces, it is necessary to differentiate the segments of these two worms. In mature segments of the beef-tapeworm the uterus contains more than fifteen lateral branches (Fig. ,N), whereas, the uterus of the pork tapeworm has fewer than thirteen lateral branches (Fig. ,M). Infection with the beef tapeworm is world-wide in distribution.

\*Pork Tapeworm (Taenia solium) (Fig. ,L and M.). Two types of human infection with this helminth may occur. The most common is infection with the adult, acquired by accidentally ingesting infective larval forms (cysticerci) with insufficiently cooked infected pork. The adults usually inhabit the small intestine. Diagnosis of infection with the adult is accomplished by recovering segments of the adult worm, or eggs, in the host's feces. It is necessary to differentiate these segments from those of the beef tapeworm (see above, "Beef tapeworm"). The less common, but more serious infection is acquired by ingesting the eggs. The larval form (cysticercus) develops from the eggs, and may encyst in vital centers with serious results. The ocular region and the brain are chosen as sites of encystment more commonly than any other parts of the body in human cysticercosis. It is entirely possible for a person infected with the adult worm, to accidentally ingest eggs from this parasitizing adult and acquire the cysticercus infection. Diagnosis of cysticerci is difficult, and is usually not attempted in the routine laboratory unless the cysts are located in superficial tissues, whence they may be excised and examined. X-rays are helpful in diagnosing calcified cysts located in vital centers. A history of infection with the adult worm may be of aid in confirming diagnosis. Both types of infection are world-wide in distribution.

\*Dwarf Tapeworm (Hymenolepis nana) (Fig. ,D). This is the most common tapeworm in the Southern United States. Infection is acquired by accidental ingestion of eggs. The adults usually inhabit the small intestine. No intermediate host is necessary in this infection, and the person harboring the infection can readily reinfect himself by accidentally ingesting eggs evacuated in his own feces. Diagnosis is based on recovery of eggs in the feces of the host. The infection is world-wide in distribution.

\*Large Intestinal Roundworm (Ascaris lumbricoides) (Fig. ,F, G, and E). This very common intestinal roundworm is acquired by ingestion of embryonated eggs. Adults usually inhabit the small intestine, and eggs evacuated in the host's feces serve as a basis for diagnosis. The laboratory worker should be able to recognize fertilized, unfertilized and decorticated *Ascaris* eggs. Decorticated eggs should not be confused with hookworm eggs (Fig. ,H and I). The parasite is world-wide in distribution.

\*Human Whipworm (Trichocephalus trichiurus, syn. Trichuris trichiura) (Fig. A). This is another very common intestinal roundworm acquired by ingestion of embryonated eggs. Adults usually live in the cecum, but may be found in other parts of the large bowel and even in the appendix. Diagnosis is based on recovery of the eggs in the host's feces. World-wide in distribution.



\*Strongyloides stercoralis, (Fig. ,K). Infection is acquired by invasion of special larval forms (Filariform larvae) through the skin, or by accidental ingestion of these larvae. In either event the larvae enter the blood stream and reach the alimentary tract indirectly. The adult males are not tissue parasites and are voided in the feces, but the females usually inhabit the walls of the duodenum and upper jejunum. The eggs are laid and hatch in the tissues. The larvae (rhabditoid larvae) migrating into the intestinal lumen and passing out in the host's feces. Sometimes these larvae, which are normally not infective, may transform into the infective type (the filariform larvae) during their transit in the intestine. These infective larvae may invade the tissues of the lower bowel producing reinfection. Diagnosis is based on recovering larvae in the host's feces. Distributed in warm moist regions throughout the world.

\*Hookworm (Necator americanus and Ancylostoma duodenale) (Fig. ,I and O). Infection is acquired by invasion of filariform larvae through the skin. The adults usually live attached to the mucosa of the small intestine. Diagnosis is based on recovering eggs in the feces of the host. Distributed in warm, moist regions throughout the world. For the most part, however, the American hookworm (Necator americanus) is found in the Western Hemisphere and in the middle and Southern two-thirds of Africa; whereas, the Old-world hookworm (Ancylostoma duodenale), is usually found in Europe and in the northern third of Africa. In Asia, the East Indies, and Australia both species of hookworm are encountered.

\*Human Pinworm or Seatworm (Enterobius vermicularis, syn. Oxyuris vermicularis) (Fig. ,N). Infection is acquired by ingestion of embryonated eggs. Eggs are usually embryonated when deposited by the female. The adults live in the cecum, appendix, and adjacent parts of the large and small intestines. The gravid females usually pass out through the anus and deposit eggs on the perianal and perineal folds. Eggs are not commonly deposited in large numbers in the bowel, and in examining for evidence of pinworm infection, ordinary fecal examination will not suffice. A special technique with an anal swab is recommended (page NIH Anal Swab). Of course, eggs or adults found in the feces also serve as a basis for diagnosis. This parasite is world-wide in distribution, and is probably much more common than surveys (which usually do not include anal swabs) have indicated.

Technic for examination of feces is given on page

Blood. \*Bancroft's Filariasis (Wuchereria bancrofti). Infection is acquired during the bites of mosquitoes infected with special larval forms. Adults normally inhabit the lymphatic vessels and the lymph glands; microfilariae probably being deposited in these places, and carried into the blood stream. Diagnosis is based on recovery of the microfilariae in thick blood-films. X-rays are helpful in chronic cases where the microfilariae may not be found. Distributed throughout the world in tropical and temperate regions.

\*Persistent Filariasis (Asymphylodermatitis perstans). Infection is acquired during the bite of the "punkie" (Culicoides) infected with special larval forms. Adults live in the body cavities and associated tissues, including mesentery, pleural cavity, etc. Microfilariae recovered in the blood stream serve as a basis for diagnosis. Distributed in Africa, South America and the East Indies.

\*Eye Worm (loa loa). Infection is acquired during the bite of the mango fly (Chrysops) infected with special larval forms. The adults move about in the subcutaneous and deeper cutaneous tissues, microfilariae being discharged into the passages produced during these migrations. Diagnosis is based on recovery of adults from the migratory tunnels, or upon the presence of microfilariae in blood films. This parasite is widely distributed in Central West Africa.

For routine laboratory examination it is probably not necessary to differentiate the microfilariae of the various species inhabiting the blood, because there is no known treatment for any of these species.

Technic for examination of blood is given in section on protozoa, page .

Tissue. \*Sparganum. Under the term "sparganosis" is grouped infection with the larval forms (Sparganum stages) of certain helminths, probably tapeworms. In most cases the adults are unknown, but the most common sparganum of man (Sparganum mansoni) is in the adult stage a tapeworm of dogs and cats. The spargana are found in the subcutaneous and ocular tissue of man where, particularly in the latter region, they may produce intense pain and tissue reaction. Infection is usually acquired by poulticing an inflamed surface of the body with the infected flesh of a cold-blooded vertebrate (e.g., frogs). Diagnosis is based on the excision of the spargana from the site of infection. The great majority of cases of human sparganosis have been reported from the Orient, but sporadic cases have appeared in other parts of the world, including the United States.

\*Hydatid Worm (Echinococcus granulosus). "Hydatid disease" is infection with the larval stage of a dog tapeworm (Echinococcus granulosus). Human infection is acquired by accidental ingestion of the eggs. The larval form (hydatid) develops from the egg and may encyst in various organs, particularly the liver and the lungs. The most accurate diagnosis of hydatid cysts is based on an intradermal reaction (the Casoni test). Hydatid infection is distributed chiefly in the sheep- and cattle-raising regions of the world, and has been reported from the United States.

\*Trichina Worm (Trichinella spiralis). Infection is acquired by eating insufficiently cooked pork which is infected with the encysted larvae. The females deposit larvae in the lymphatics of the duodenum and jejunum, probably also in the mesenteric veins. Larvae are carried to all parts of the body, and migrate out and encyst in striated muscles. Diagnosis is usually based on an intradermal test, or upon recovery of larvae in excised muscle. Distributed chiefly in the United States and Central Europe. Rarely reported from parts of Africa and South America.

\*Ancylostoma braziliense. This species, Ancylostoma braziliense, is a tropical hookworm of dogs and cats. On contact with human skin, the larvae enter; but since they cannot penetrate the blood vessels, they migrate in the skin layers producing a "creeping eruption". Many human cases of creeping eruption have been reported from the Southern United States, but cases are likely to be encountered wherever animals harbor the adult hookworm.



\*Convolutad Filaria (Onchocerca volvulus). Infection is acquired during blood-meal of the black fly (Simulium) infected with certain larval forms. Adults usually live in tumors in subcutaneous or connective tissues, and deposit larvae inside of these "nodules". Diagnosis is based on demonstration of adults or microfilariae from excised nodules; or when no palpable nodules are present, microfilariae from the skin or conjunctive. Distributed in Africa, Central America (particularly Western Guatemala) and Southern Mexico.

\*Guinea Worm (Dracunculus medinensis). Infection is acquired by ingestion of infected small crustaceans (Cyclops) in raw drinking water. Adults develop in the viscera or in the subcutaneous tissue, and when mature migrate to a position just under the skin. A blister develops on the skin just above the head of the worm; after a few days this blister bursts, and on contact with fresh water, the worm deposits larvae into the water through the ruptured blister. Diagnosis is based on finding the worm under the skin when local lesions have developed. Distributed in Africa, Southern Asia, the West Indies, the Guianas and Brazil. Sporadic cases have appeared in other localities.

Regarding examination of tissue for parasites see page ., Tissue.

Urine. \*Vesical Blood Fluke (Schistosoma hematobium) (Fig. ,3). Infection is acquired in a similar manner to Manson's Blood Fluke. Adults of this species inhabit the portal blood and vesical plexuses. Eggs found in the urine, rarely in feces, constitute the basis for diagnosis. Distributed extensively in Africa.

Technic for examination of urine is given on page .

Sputum. \*Oriental Lung Fluke (Paragonimus westermani) (Fig. ,1). Infection is acquired by consumption of raw crabs and crayfish harboring infective larval cysts. The adults usually inhabit the bronchioles of the lung although they may be found in other sites. Diagnosis is usually based on recovery of the eggs in blood-flecked sputum, but eggs may also be recovered in the feces, and rarely in cutaneous lesions. For the most part this fluke is oriental in distribution.

Technic for examination of sputum is given on page .

#### METHODS OF EXAMINATION

Feces. Ordinary Smear. In many cases of helminthic infection an ordinary fecal smear examined under the microscope will show eggs or larvae of the offending parasite. For details of this technic see section on protozoology, page .

Zinc Sulfate Centrifugal Flotation Technic. Many cases of helminthic infection are sufficiently light that no eggs or larvae will be recovered in an ordinary fecal smear. It is then necessary to employ the Zinc Sulfate Centrifugal,

Flotation Technic. It is a good policy to examine each stool by this technic, because the presence of an helminthic infection by an ordinary smear is no indication that other parasites are absent. For details of this technic see page , Zinc Sulfate Centrifugal Flotation Technic.

NIH Anal Swab. In pinworm infection eggs are more commonly deposited on the perianal folds than in the feces, therefore, swabbing and mild scraping of these parts yield eggs even when the feces is negative. The NIH Anal Swab is the most efficient anal swab and scraper yet devised. Its preparation follows:

1. Insert a glass rod through a rubber stopper so that approximately one inch protrudes from the larger end of the stopper.
2. Using a rubber band, secure a cellophane square to the other end of the rod as shown in the accompanying diagram (Fig. ). (The rubber bands should be about 2mm. wide, and may be from rubber tubing having a 3 mm. bore and walls 2 mm. thick.)
3. Fit the swab into the test-tube housing as shown in the diagram.

Before describing the actual swabbing procedure, a few suggestions, regarding the use and examination of the swab may be in order. First, the swabbing should be done in the morning immediately after the patient arises, before he has bathed or defecated. Second, the swab is to be used dry. Third, certain defects in cellophane resemble pinworm eggs in outline, and the worker should guard against this possible source of error in diagnosis.

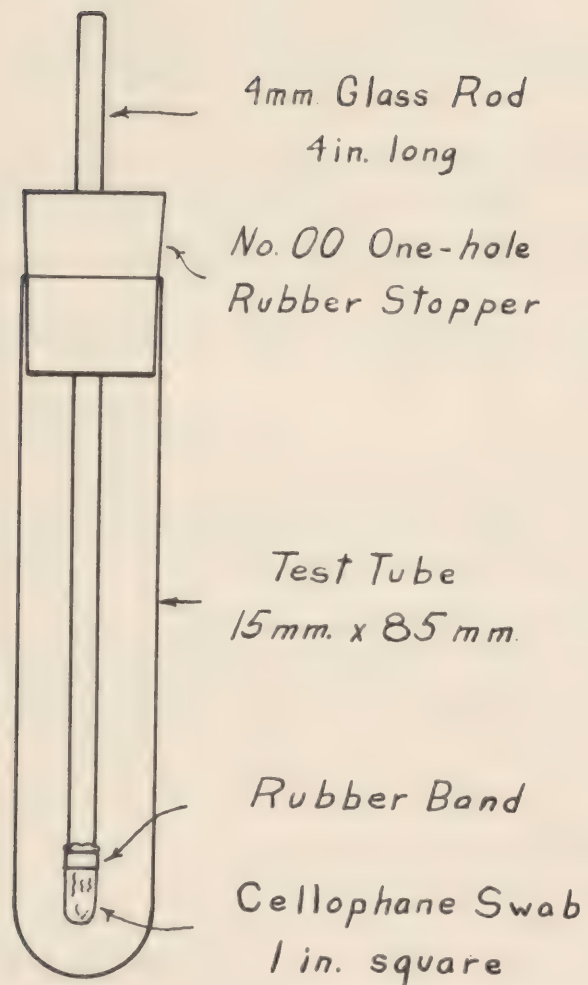
Swab and examination is as follows:

1. Stroke the cellophane-covered tip firmly, with an outward motion, over the perianal folds and across the anal opening.
2. Release the cellophane square by sliding the rubber band towards the rubber stopper.
3. Mount the cellophane square in water on a microscopic slide and examine under a microscope.

Tapeworm Segments. It is necessary to differentiate segments of beef or pork tapeworms passed in the feces. This is done as follows:

1. Clean and relax the segments by shaking in physiological saline.
2. Place the specimen between two glass slides, and press it flat.
3. Hold it up to a strong light (so that the light shines through it), and count the number of lateral branches of the uterus. (Since these lateral branches sub-divide, they are to be counted where they arise from the main part of the uterus)





The NIH Anal Swab





Blood. Thick Films. Thick films are very useful in demonstrating micro-filariae. For details of this technic see page , Fecal Smear.

Tissue. There are several specialized technics employed in the diagnosis of tissue parasites, most of which are not entirely satisfactory. Need for these technics is not very common, but when necessary, the technic is probably best performed by the medical officer in charge. Details of these technics are available in standard texts.

Urine. In cases of heavy infection with the vesical blood fluke eggs may be found in the urine, particularly in the last portion passed. The technic follows:

1. Have the patient pass urine into a urinalysis glass. (This should especially include the last portion of urine voided.)
2. Let the material settle for 15-20 minutes.
3. Take up a small portion of the sediment in a pipette and place it on a microscopic slide.
4. Examine under the microscope.

In cases of light infection this procedure may yield negative results. It is then necessary to centrifuge a representative portion of the urine for 1-2 minutes, and examine some of the sediment under a microscope.

Sputum. In many cases of suspected helminthic infection of the respiratory passage, examination of the sputum is necessary. The technic follows:

1. Rinse the mouth thoroughly with diluted hydrogen peroxide.
2. Pass sputum into a jar.
3. Transfer small bits of sputum, particularly blood-flecked portions to a microscopic slide.
4. Examine under a microscope.

#### TRANSMISSION OF SPECIMENS

From time to time it is necessary to send specimens to other laboratories--sometimes for identification; sometimes for study purposes. In all such cases, complete notes should accompany the material, and should include such data as locality, host, date, collector's name, number of specimens obtained, condition of the specimens, tissue, organ, or medium from which recovered, and other pertinent information. Material should be treated for shipment as indicated below.

Eggs. Feces containing eggs may be diluted with water and agitated until an even mixture is obtained. To this mixture is added an equal volume of steaming (20°C.) 10 per cent formalin. This will also fix any nematode larvae present.

Larvae and Adults. Larvae and adult helminths should first be shaken in physiological saline. This cleans and relaxes the specimens. They may now be fixed by adding an equal volume of steaming (80°C.) 5 per cent formalin to the saline containing the worms.

Pathological Tissues. Pathological tissues may be fixed in 10 per cent formalin, or if it is available, Zenker's fluid.

Intermediate hosts. Intermediate hosts may be fixed and preserved in 70 per cent alcohol.

Specimens should always be carefully packed to avoid breaking or spilling of the contents. Containers should be filled with the preserving liquid to avoid breaking of the specimens if the package is handled roughly. Jars and glass vials packed separately in a box with excelsior, shredded paper or cotton, and marked "Fragile" will usually survive.

#### BIBLIOGRAPHY

Among the more important texts of interest to the laboratory worker in helminthology are:

- Faust, E. C., "Human Helminthology", Phila., 1939.
- Craig, C. F., and Faust, E. C., "Clinical Parasitology", Phila., 1940.
- Manson-Bahr, P. H., "Manson's Tropical Diseases", Baltimore, 1940.



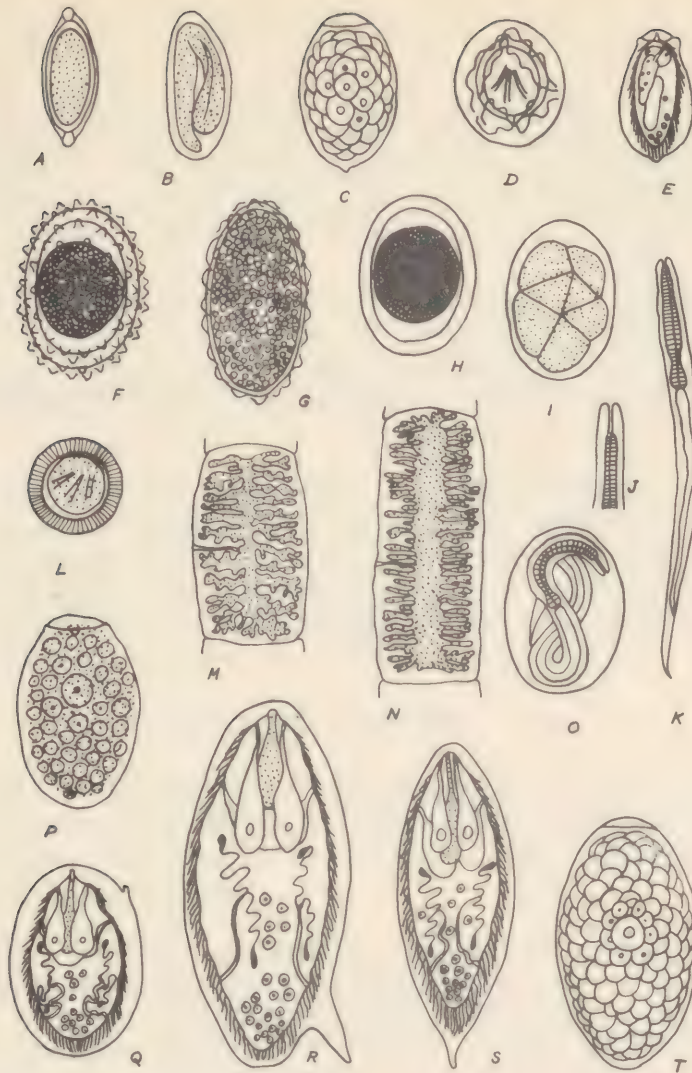


Fig. Helminths. Diagram of diagnostic material. A, egg of the whipworm (*Trichocephalus trichiurus*); B, egg of the pinworm (*Enterobius vermicularis*); C, egg of the broad fish tapeworm (*Diphyllobothrium latum*); D, egg of the dwarf tapeworm (*Hymenolepis nana*); E, egg of the Chinese liver fluke (*Clonorchis sinensis*); F, G, H, fertilized, unfertilized, and decorticated eggs respectively of the large intestinal roundworm (*Ascaris lumbricoides*); I, egg of hookworm (*Necator americanus* or *Ancylostoma duodenale*); J, fore-part of rhabditoid larva of hookworm showing long buccal cavity. Compare with short buccal cavity of rhabditoid larva of *Strongyloides stercoralis*, K; L, egg of either the pork tapeworm (*Taenia solium*) or the beef tapeworm (*T. saginata*); M, N, gravid segments of the pork and beef tapeworms respectively, showing difference in the number of uterine branches—fewer than fifteen in the pork tapeworm; more than fifteen in the beef tapeworm; O, egg of hookworm showing fully developed embryo. Such eggs are commonly found in constipated stools of hookworm patients. P, egg of the Oriental lung fluke (*Paragonimus westermani*); Q, egg of the Oriental blood fluke (*Schistosoma japonicum*); R, egg of Manson's blood fluke (*S. mansoni*); S, egg of the vesical blood fluke (*S. hematobium*); T, egg of the sheep liver-fluke (*Fasciola hepatica*) or the large intestinal fluke (*Fasciolopsis buski*). Approximate magnifications, E X600; M and N X3; all others X300. Compiled and modified from various sources.





## THE PROTOZOA

The protozoa are very small animals, the body consisting of a single cell. Due to their small size they are always studied under a microscope. They are important to the medical soldier because some species of protozoa inhabit various parts of the human body and cause disease. Amoebic dysentery, malaria and sleeping sickness are just a few of the diseases caused by these organisms.

Compared to bacteria, which are plant germs, protozoa (or animal germs) move about more rapidly; their internal structure is more easily seen, and is usually diagnostic of the species. They are grown only with difficulty on artificial culture media and are usually crowded out by bacteria when these latter forms contaminate the cultures.

Although protozoal diseases are more widespread in tropical countries, they are also common in temperate zones. Regardless, they are of utmost importance to the medical soldier because of their epidemic tendencies, and the fact that a large number of military personnel may have served, or are now serving, in areas where protozoal infections are common. Many of these men may develop chronic cases of disease, and in this "carrier" state serve to infect many others.

### THE INTESTINAL PROTOZOA

The Amoebae. Included among the protozoal parasites of man are the amoebae, which resemble each other in moving about by "false feet" (pseudopodia). Of the half dozen species inhabiting the intestinal tract of man, only one has been proven to be pathogenic - Endamoeba histolytica.

Endamoeba histolytica. Although amebiasis (infection with E. histolytica) is more commonly found in tropical and near tropical regions, it has reached epidemic proportions in temperate climates (Chicago World's Fair, 1935), and is world-wide in distribution. It is estimated that in the United States alone 6 to 12 millions of persons harbor this parasite, and incidence of 5-10 per cent.

Accurate diagnosis of E. histolytica is absolutely essential, because of the seriousness of the disease. Furthermore, the erroneous diagnosis of one of the harmless amoebae as E. histolytica would subject the patient to a needless long and severe treatment. The other intestinal amoeba most commonly confused with E. histolytica is E. coli. Chart \_\_\_\_\_ and Fig. \_\_\_\_\_ will enable the laboratory worker to differentiate the several intestinal species.

Other Intestinal Protozoa. In addition to the amoebae, other intestinal protozoa may occasionally be encountered. Since these forms are either harmless or only rarely found, they are mentioned only in passing. For the most part these organisms are sufficiently different in appearance as not to be confused with Endamoeba histolytica. For details regarding these forms standard texts \* are available.

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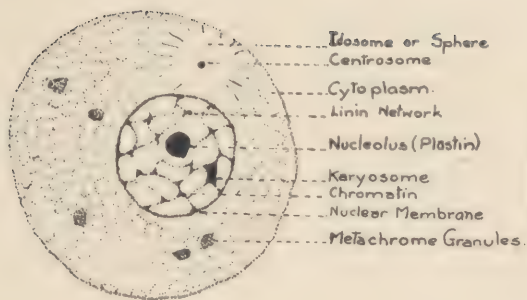
\* See bibliography

One of these protozoa is an amoeba, Dientamoeba fragilis. This amoeba is relatively rare, and is generally conceded to be non-pathogenic.

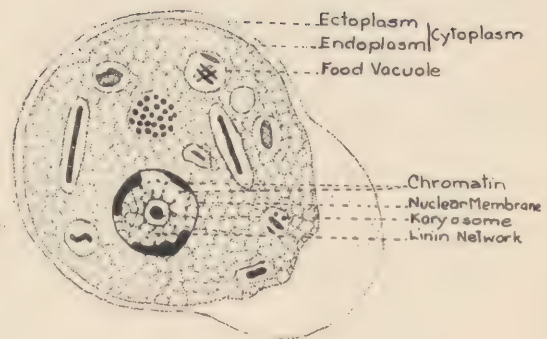
Among the intestinal flagellates are Chilomastix mesnili, Trichomonas hominis and Giardia lamblia. Some investigators are of the opinion that these species, particularly the latter two are pathogenic. Until any of these flagellates have been definitely proven to be the cause of disease, however, it is the part of wisdom to regard them as harmless.

Balantidium coli is a pathogenic ciliate inhabiting the intestine of man. It produces a dysenteric condition very similar to that caused by Endamoeba histolytica. However, since human infection with E. coli is only very uncommonly encountered, this parasite will not be discussed further here.

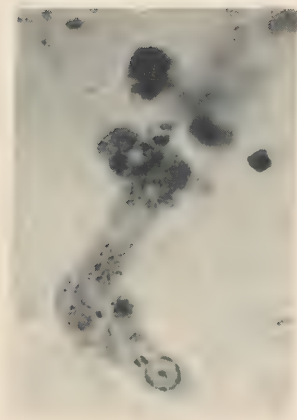




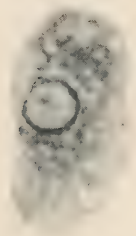
Metazoan Cell



Protozoan Cell



A.



B.

Trophozoites of Amoebae  
 A. *Endamoeba histolytica*  
 B. *Endamoeba coli*





TABLE 86.--CHARACTERISTICS OF THE AMOEBAE OF MAN.

## Stained Trophozoites.

	<u>E. histolytica.</u>	<u>E. coli.</u>	<u>E. mann.</u>	<u>I. butschlii.</u>	<u>D. fragilis.</u>
Average size	20 to 35 $\mu$	15 to 30 $\mu$	6 to 10 $\mu$	9 to 13 $\mu$	3 to 12 $\mu$
Nuclear membrane (stains faintly or not at all)	Lined with minute, fairly even sized grains of chromatin which stain deeply.	Lined with coarse irregularly sized grains or bars of chromatin which stain deeply.	Chromatin on nuclear membrane in thin line and stains poorly.	A few poorly staining, widely separated, chromatin grains on nuclear membrane.	Chromatin on nuclear membrane in thin line and stains poorly. Nucleus frequently double.
Karyosome	Short rod or globule of small diameter, centrally suspended within the nucleus. Regular outline. Stains deeply and uniformly.	Short rod or ball or irregular outline, usually eccentric. Diameter greater than that of <u>E. histolytica</u> . Stains deeply and uniformly.	Very large, central or eccentric, composed of 1, 2 or more deeply staining masses in a lighter staining matrix. Outline often irregular and oblong.	Similar to that of <u>E. mann</u> but larger and more apt to contain a poorly staining central portion. Causes the nucleus to appear like an eye with a widely dilated pupil.	Composed of several minute deeply staining, discrete grains.
Linin network (stains faintly or not at all)	Contains no chromatin grains between the karyosome and nuclear membrane.	Sometimes contains grains of chromatin. Region just without karyosome halo often appears cloudy after staining.	It is not often discernible. Consists of a few short lines from the karyosome halo to the nuclear membrane. (Karyosome usually the only structure visible in the nucleus.	Like a web when defined by an excellent stain.	Not demonstrable.

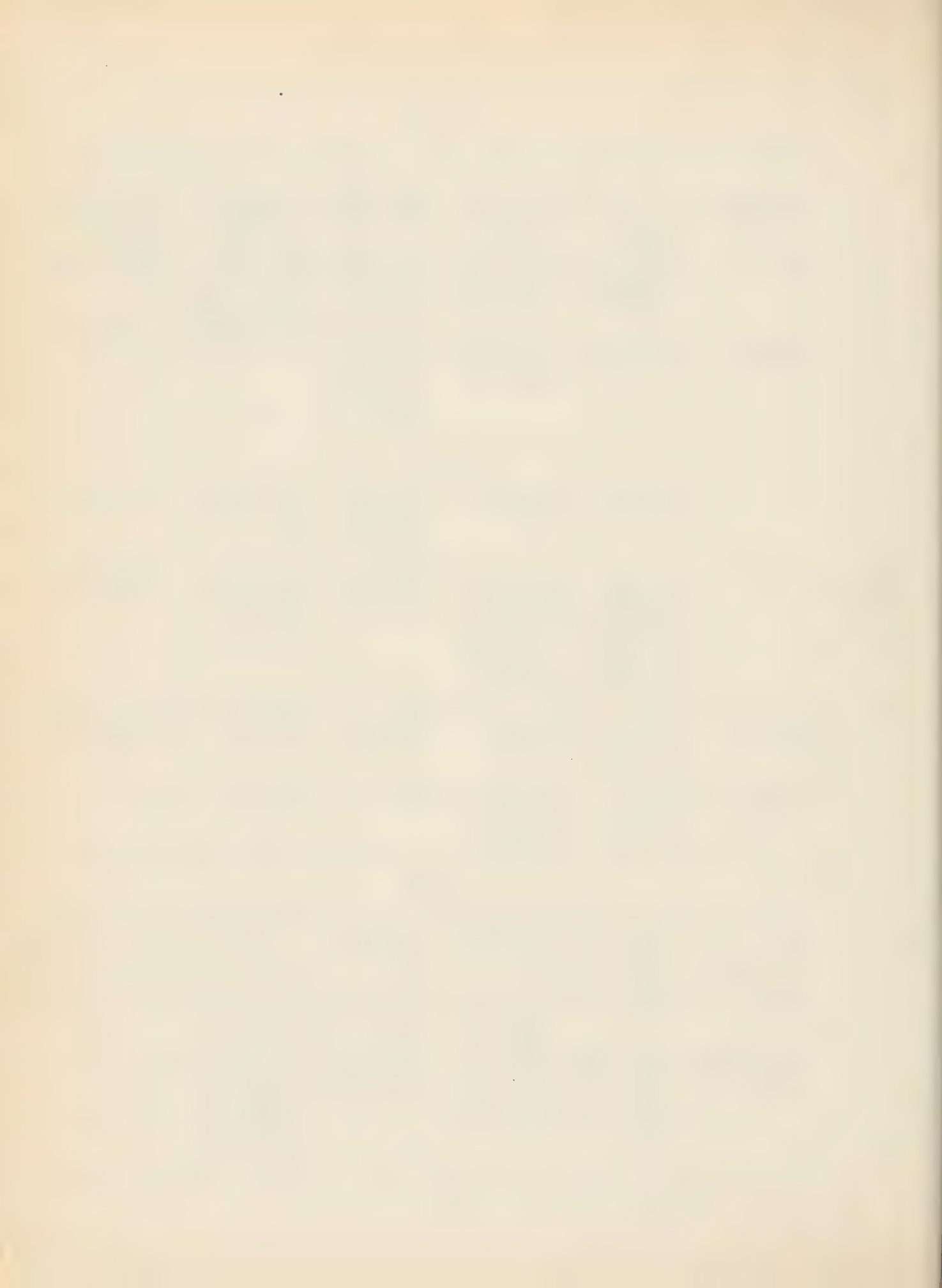
## The Living Trophozoites.

Motion	Active progression in a definite direction. Form is elongated in motion.	Most strains are not actively progressive but merely change in conformation.	Some organisms like that of <u>E. histolytica</u> (except the amoeba is very small) but the majority merely change in form and do not move progressively.	Many strains in culture like that of <u>E. histolytica</u> . The majority, however, are like <u>E. coli</u> .	Slow indefinite progression or merely change in conformation.
Pseudopodia	Finger-like with smooth outline when not in progressive motion. Ectoplasm is clear, glass-like and easily discernible. When in progressive motion the ectoplasm may not be clearly differentiated. One-third to one-quarter of the parasite is ectoplasm.	Usually blunt, but it may be like <u>E. histolytica</u> . The ectoplasm is usually not clearly differentiated. One-quarter to one-fifth of the parasite is ectoplasm but it is often poorly differentiated from the endoplasm even when the amoeba is in motion.	Like <u>E. histolytica</u> . One-half to one-third of the parasite is ectoplasm and is easily differentiated.	Like <u>E. histolytica</u> or very broad with coarsely indented outline. One-half to one-third of the parasite is ectoplasm and is easily differentiated.	Often comprises one-half of the organism. Outline is often indented.
Color	Faint green.	Gray.	Gray.	Faint green.	Gray.
Visibility of nucleus (oil-immersion lens)	Usually difficult to visualize except when the nucleus passes into the pseudopodia and is contrasted against the clear ectoplasm.	Quite clear. It is much more readily seen than that of <u>E. histolytica</u> .	The karyosome may be defined with ease.	The karyosome may be defined with ease.	Difficult to distinguish from ingested bacteria.
Endoplasmic inclusions of diagnostic significance.	RBC blood cells are typical and diagnostic. Degenerated and culture forms contain bacteria.	A voracious feeder but usually does not ingest red blood cells. Bacteria and starch grains are the principal inclusions.	Bacteria.	Bacteria.	Bacteria.

Cystic Stage.<sup>1</sup>

	5 to 20 $\mu$	10 to 30 $\mu$	5 to 8 $\mu$	9 to 15 $\mu$
Average size				
Nuclei number	1 to 4, rarely more. Mature cysts contain 4 nuclei.	1 to 8, rarely more. Mature cysts contain 8 nuclei.	1 to 4, rarely more. Mature cysts contain 4 nuclei.	1 or 2.
Visibility of nuclei in the unstained living state.	Poor but discernible with the oil-immersion lens.	Good.	Good.	Good.
Shape	Generally spherical or nearly so.	Generally longer than broad and one side may be less curved than the other.	Irregularity of shape is common. Generally oval.	Greatest irregularity of shape and outline is common.
Reserve food inclusions (these disappear in old specimens and are not constant in young cysts).	Bar-shaped chromatoid bodies in 0-90 per cent of cysts. Sometimes a small amount of glycogen is present in young cysts. It is diffuse and stains a light brown with iodine.	Acicular chromatoid bodies present in about 10 per cent of cysts. A large amount of glycogen may be present and push the nucleus against the cyst wall.	Small granules or masses of volutin and glycogen may be present. Neither is characteristic.	Masses, grains or rods of volutin may be present but these are not characteristic. The glycogen, almost invariably present, is large in amount, smoothly outlined and stains a deep brown with iodine. This iodine body is characteristic and diagnostic.

<sup>1</sup> Cystic stage of E. fragilis is unknown.





# GRAPHIC DIFFERENTIATION OF AMOEBA

	<i>E. histolytica</i>	<i>E. coli</i>	<i>I. butschlii</i>	<i>E. nana</i>	<i>D. fragilis</i>	<i>E. gingivalis</i>	<i>A. proteus</i>
Trophozoites							
Pre-cystic forms							
Cystic forms							





# Cystic Stage

	<u>E. histolytica</u>	<u>E. coli</u>	<u>E. nana</u>	<u>I. butschlii</u>
average size	7 to 15 u	10 to 30 u	5 to 15 u	7 to 15 u
nuclei number	1 to 4, rarely more. Mature cysts contain 4 nuclei.	1 to 8, rarely more. Mature cysts contain 8 nuclei.	1 to 4, rarely more. Mature cysts contain 4 nuclei.	Usually 1. Rarely 2.
visibility of nuclei in the unstained living state.	Poor but discernible with the oil-immersion lens.	Good.	Good.	Good.
shape	Generally spherical or nearly so.	Generally longer than broad and one side may be less curved than the other.	Irregularity of shape is common. Generally oval.	Great irregularity of shape and outline is common.
reserve food inclusions (these disappear in old specimens and are not constant in young cysts).	Star-shaped chromatoid bodies in 0-90 per cent of cysts. Sometimes a small amount of glycogen is present in young cysts. It is diffuse and stains a light brown with iodine.	Acicular chromatoid bodies present in about 10 per cent of cysts. A large amount of glycogen may be present and push the nucleus against the cyst wall.	Small granules or masses of volutin and glycogen may be present. Neither is characteristic.	Masses, grains or rods of volutin may be present but these are not characteristic. The glycogen almost invariably present is large in amount, smoothly outlined and stains a deep brown with iodines. This iodine body is characteristic and diagnostic.

## GENERAL METHODS USED IN THE DIAGNOSIS OF THE INTESTINAL PROTOZOA

### COLLECTION OF SPECIMENS

General Directions and Precautions. The specimen should be collected directly in clean, covered receptacles (bed pans, swabs in test tubes containing  $\frac{1}{2}$  cc. of physiological saline, syringes, bottles or droppers) preferably sterilized by heat. These receptacles should not be sterilized by chemical disinfectants as protozoa in the vegetative stage are easily killed and quickly autolyze in the presence of only small amounts of such chemicals. If the receptacles are not properly cleaned and sterilized, there is always the possibility of introducing free-living protozoa into the specimen and thus confusing the picture. All specimens should be examined at the earliest possible opportunity after collection. These protozoa degenerate rapidly and the possibility of an accurate diagnosis diminishes as the time between collection and examination of the specimen increases.

If the pathological lesions are in the rectum or sigmoid, specimens may be obtained by the physician by means of the proctoscope or the sigmoidoscope and sent to the laboratory for examination. These are more apt to yield protozoa than the passed feces. However, because of the attending discomfort and perhaps pain to the patient, this method should be used only after it has been demonstrated that the feces are negative.

It is practically impossible to find protozoa in a stool after an oil cathartic, e.g., castor oil, mineral oil, or following a barium meal. Specimens collected by means of an enema are also unsatisfactory. Therefore, in the event any of the above has been used, examination for protozoa in the feces should be delayed for at least 72 hours.

The portions of a stool most likely to contain parasitic protozoa are those showing blood or mucus. In formed stools, small flecks of mucus or mucus and blood can always be found on the surface of the specimen. In semifformed or liquid stools if the specimen is examined carefully, mucus and blood can also be found.

Formed stools usually contain only cysts or precysts of protozoa. Semifformed and liquid stools will ordinarily contain only vegetative forms, and it is these types of stools that best afford an opportunity to diagnose a protozoan infection. If the patient is not already passing such a stool, and it is necessary to rule out the possibility of a parasitic protozoan infection, give a saline cathartic and collect the second or third liquid stool passed for examination.

In patients infected with intestinal protozoa, the number of organisms present on an infected surface at any given time depends upon the type of pathology produced, the bacterial flora present and the resistance of the host. Because of this multiplicity of governing factors, protozoa usually appear in showers; they may be present in great numbers one day and relatively scarce the next. Therefore, to arrive at a satisfactory diagnosis in questionable cases, repeated examinations should be made on at least three consecutive days.



## PREPARATION OF THE SPECIMEN FOR MICROSCOPIC EXAMINATION

**Examination of Fresh Material.** These specimens should be kept in the original receptacle used for collection until examined. The material should be kept at a temperature of 37°C. as all vegetative organisms in this group are quite sensitive to chilling, and are also rapidly killed by temperatures of 45°C. or higher. Since drying also affects these organisms the specimen should have its original moisture content when presented for examination.

**Fecal Smear.** The general procedure for examination of dysenteric, diarrheic or mushy stools, where the vegetative forms (trophozoites) are most likely to be found, follows: Warm a clean slide so that it feels comfortable when touched by the back of the hand. Then secure a small piece of mucus or mucus and blood from the fresh specimen by means of a wire loop or wooden applicator and thoroughly emulsify it in one drop of physiological saline on the middle of the slide. Now take a clean number one cover slip between the thumb and forefinger of the right hand, contact the slide with one edge of the cover slip near the drop but not touching it, push the slip along the surface of the slide until its edge contacts the drop, rock it slightly from side to side to allow a portion of the fluid to come under the edge of the slip, then let the cover slip drop from between the fingers allowing it to fall on the side. The fluid portion of the drop on the slide will then automatically be drawn by capillary attraction under the slip, while the solid particles will be excluded. This method insures a thin even preparation of not too great a density (when newsprint is viewed through it the words are legible) and insures even apposition of the cover slip to the slide. The preparation is now ready for examination, but if it is to be kept on a warm stage for any period of time it should be ringed with vaseline.

**The Zinc Sulfate Centrifugal Flotation Technic.** In formed or semi-formed stools, where cysts are more likely to be found, the Zinc Sulfate Centrifugal Flotation Technic is very valuable. Ordinary fecal smears should also be done, however, since they give some indication of the intensity of the infection, the zinc sulfate method having its greatest value in enabling the worker to discover light infections.

1. Prepare a fecal suspension by emulsifying one part stool specimen (about the size of a pecan) in 10 parts luke warm water.
2. Stain 10 cc. of this emulsion through two layers of wet cheesecloth into a Wassermann tube.
3. Centrifugalize for 45 - 60 seconds at approximately 2500 RPM, pour off the supernatant fluid and add 2 - 3 cc. of water. Then break up the sediment and repeat the above centrifugalizing and discarding the supernatant fluid 3 or 4 times.
4. After pouring off the last supernatant fluid add 3-4 cc. zinc sulfate (33 per cent solution), break up the packed sediment and add enough zinc sulfate solution to fill the tube to about  $\frac{1}{2}$  inch from the rim.
5. Centrifugalize the tube for 45-60 seconds at top speed.
6. Remove several loopful of the material floating on the top surface film to a clean slide. Add one drop of iodine stain and a cover slip.



**Iodine Stain.** This is a temporary stain used for quick diagnosis on fresh material, and must be made up fresh every ten days or it will not stain properly. It is prepared by mixing the following:

Iodine. . . . .	.5 gram
Potassium iodide. . . . .	1.0 gram
Distilled water . . . . .	100 cc.
Glacial acetic acid . . . . .	1.0 cc.

After finding trophozoites or cysts and studying them in the fresh wet unstained mount, the nuclear details can be clearly visualized and studied microscopically if the cover slip is raised at one edge and a drop of the iodine stain is thoroughly mixed with the contents under the slip.

**Culture Specimens.** Culture specimens are prepared for microscopic examination exactly as fecal smears above except that because of their fluid nature they are not mixed with physiological saline prior to applying the cover slip.

### SPECIAL STAINING

**Fixation.** The specimen to be fixed should be fresh, and the organism should not show any evidence of degeneration. If the specimen is old and the organisms are degenerated, good fixation and staining will be impossible. The best fixative for protozoa in general laboratory use is prepared as follows:

#### Schudinn's Solution

Mercuric chloride, saturated solution in physiological saline. . . . .	65 cc.
Ethyl alcohol, 95% . . . . .	35 cc.
Glacial acetic acid. . . . .	5 cc.

The glacial acetic acid and the ethyl alcohol should be added to the mercuric chloride solution just before use.

The general procedure in fixing protozoan specimens follows: If the material to be fixed is not in the liquid state, mix enough physiological saline with it to form a thin watery solution. Now rub well into one half of the surface of a clean slide one small drop of fresh egg white or normal horse serum, then put one large drop of the specimen on this surface and spread it evenly. Allow the preparation to dry only until the fluid portion of the specimen will no longer run when the slide is tilted (the film will still be moist), then drop the slide, film-side down, into 50 cc. of the above fixing solution contained in a Petri dish. After ten minutes turn the slide over or place it in a Coplin jar three-fourths full of the same fixative and allow it to remain for one to two hours.

**Amoeba Trophozoites.** Slide preparations for fixing and staining these organisms are made in the same way except that as soon as the drop of fecal material is placed on the slide a cover slip is dropped on it. The preparation is allowed to set for three minutes, and then placed in a Coplin jar. The fixative is then carefully added and is allowed to act for 2 hours. After fixation the cover slips are removed from each specimen and the slides are then ready to be carried through the routine of staining. This method of putting the cover slip on the preparation makes it possible to inspect the slide for amoebae prior to fixation



and in addition is a great help in keeping the amoebae on the slide during fixation.

After fixation, the preparations should be rinsed in tap water (i.e., water in another Coplin jar, not running water), and then placed in 70% ethyl alcohol. They may then either be transferred to 95% alcohol for preservation, or stained immediately.

Staining. The most generally useful staining process for permanent preparations of protozoa is an iron-hematoxylin technic. Details follow:

Methods of Staining Smears on Glass Slides. The fixed and washed slide specimens prepared as outlined previously are carried through the successive steps in staining as follows:

1. Immerse for 10 minutes in 70% ethyl alcohol.
2. Immerse for 10 minutes in 70% ethyl alcohol to which has been added sufficient iodine stain to produce a light mahogany color.
3. Immerse for 10 minutes in 70% alcohol.
4. Mordant in the following solution:
 

Iron alum (ferric ammonium sulfate) 4% aqueous solution. . . . .	1 part
Ethyl Alcohol, 50%. . . . .	10 parts
5. Immerse for 5 minutes in 70% ethyl alcohol.
6. Stain for 24 hours in Heidenhain's iron hematoxylin, prepared as follows:

Hematoxylin. . . . .	1 gram
Ethyl alcohol, 95% . . . . .	10 cc.
Distilled water. . . . .	90 cc.
Thymol. . . . .	one crystal

Dissolve the hematoxylin in the alcohol, add the distilled water and thymol, then allow to ripen for one month in a clear glass-stoppered bottle exposed to the sun.

7. Remove from stain and wash in two changes of tap water.
8. Destain in the following:
 

Iron alum, 2 to 4% aqueous solution. . . . .	1 part
Ethyl alcohol, 50%. . . . .	10 parts

Differentiate by agitating each slide separately in the above solution until a light grey to blue tinge predominates; control the exact point by observing the staining definition of the organism under the microscope every few minutes.

9. Rinse in tap water, then wash for 10 minutes each in three separate dishes of 70% ethyl alcohol.

10. Begin dehydration by two changes of 95% ethyl alcohol for 5 minutes each.

11. Complete dehydration by two changes of absolute ethyl alcohol for 10 minutes each.

12. Replace the absolute alcohol in the specimen by two changes of xylol for 10 minutes each.

13. Mount in Canada balsam.

The specimen should never be allowed to dry at any stage in the technique as it causes the organisms to shrink and become distorted in shape.

If the destaining agent is not thoroughly washed out of the specimen it will fade the stain.

If the specimen is not properly dehydrated before clearing in xylol, the xylol will become milky and the slide when viewed microscopically after mounting in balsam will appear blurred.

Method of Staining Protozoa in Bulk. In the staining of mucous surface protozoa of man there are almost as many variations in staining technique as there are workers in the field. The chief difficulties encountered in all of the better techniques of staining are: (1) keeping the organisms on the slide or cover slip during fixation and staining, (2) keeping the organisms free from distortion and clear from the debris so that their internal structures are not obscured, (3) carrying out proper differentiation of the internal structures of the organisms at the time of destaining, (4) having a sufficient number of well stained organisms on the slide after staining so that a diagnosis will not have to be made on a few more or less atypical organisms.

The following staining method not only satisfies the above requirements, but also affords a method of concentration, thereby making it possible to secure satisfactory stains from specimens containing very few organisms that could not be stained by other methods. The method of staining differs from other standard hematoxylin staining methods only in that the organisms are not fixed and stained on slides or cover slips, but are fixed and stained in bulk. The organisms are concentrated and then carried through the steps of fixation and staining in 50 cc. centrifuge tubes. Very few of the organisms present in the original specimen are lost during fixation, staining and mounting. The organisms are natural and lifelike and are not distorted by the reagents or manipulations used.

Precautions. There should be no delay in concentration, examination and fixation of tryphozoites after they have been secured from the patient. Needless to say degenerated organisms will not take a satisfactory stain.

Destaining must be checked by frequent microscopic observations of the organisms being differentiated. Care should be exercised to carry the destaining to the point that there is sharp differential detail between the nuclear structures and the cytoplasm. The common tendency with this technique is not to carry the



destaining far enough. The organisms should be checked for structural detail after the acid destain has been neutralized and if there has been insufficient destaining they should be carried back into acid alcohol and further destained until the desired degree of differentiation has been secured.

The stained material must be completely dehydrated before clearing in xylol.

Discard the supernatant fluid decanted off after centrifugalizing, in each step of the procedure, or if the alcohols and stains are to be used over again, filter through a Berkefeld N filter as there is danger of carrying organisms in used alcohols and stains to the next case subsequently stained.

The material being stained may be left for 24 to 48 hours additional time in any step of the staining procedure with the exception of the fixing solution and the acid alcohol destaining reagent. This allows the staining procedure to be carried out without interfering with the routine work in a laboratory.

**Concentration and Fixation.** Fecal specimens. Thoroughly emulsify 20 cc. of feces in 200 cc. of warm (37°C.) physiological saline in a settling flask or tall narrow cylinder; allow to stand for five minutes and then decant the supernatant fluid into two 50 cc. centrifuge tubes. Centrifugalize the material at approximately 1850 RPM for five minutes, then decant off the supernatant fluid, save the precipitated residue of one tube for fresh examination and to the tube containing the other precipitate add 25 cc. of fresh Schaudinn's fixing solution. Thoroughly mix the precipitate and the fixative and allow the mixture to stand for at least one hour, preferably 24 hours.

**Cultures and Other Liquid Specimens.** Pipette the fluid containing the organisms directly into a 50 cc. centrifuge tube and then centrifugalize and fix the material as indicated above.

**Staining.** (Between each step in the subsequent procedure the material is centrifugalized at 1850 RPM for 5 minutes, then the supernatant fluid is decanted off and the next solution added to the precipitated residue which is then thoroughly emulsified by rotation or stirring.)

1. Wash the fixed material two times with distilled water.
2. Wash 10 minutes with 70% ethyl alcohol (containing enough Gram's iodine to give it a light brown color. For preparation of Gram's iodine see section on bacteriology.)
3. Wash 10 minutes with 70% ethyl alcohol.
4. Stain by adding Harris' hematoxylin for from 1 to 24 hours.
 

Hematoxylin. . . . .	1 gram
Ethyl Alcohol, 95% . . . . .	10 cc.
Dissolve the hematoxylin in the alcohol.	
Alum (ammonium or potassium) . . . . .	20 grams
Distilled water. . . . .	200 cc.



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Dissolve the alum in the water by the aid of heat, and add the hematoxylin solution. Bring the mixture to a boil as rapidly as possible and then add one-half gram of yellow oxide of mercury. The solution at once assumes a dark purple color. As soon as this occurs, remove the vessel containing the solution from the flame and cool rapidly by plunging into a basin of ice water. As soon as the solution is cool it is ready for use. The addition to the stain of glacial acetic acid to a concentration of 4 per cent is supposed to increase the provision of the nuclear staining.

5. Wash with tap water.

6. Destain by adding about 20 cc. of acid alcohol (1% HCl in 70% ethyl alcohol) to the stained precipitate in the centrifuge tube. Mix the precipitate and the destaining solution and occasionally stir the mixture with a wooden applicator stick. From time to time check the progress of destaining by taking one drop of the mixture, placing it on a slide, applying a cover slip and then observing the progress of the nuclear differentiation of the organisms under the high dry power of the microscope. The organisms will be fairly easy to find in the average case and destaining should be allowed to go on until the cytoplasm is practically colorless and the nucleus stands out sharp and clear. As soon as the desired definition has been obtained, add sufficient ammonia water (5 drops  $\text{NH}_4\text{OH}$  in 50 cc. of distilled water) to neutralize the acid alcohol and turn the solution bright blue.

#### CULTURAL METHODS

Culture Media. The Boeck-Drbohlav Medium. The Boeck-Drbohlav medium is the one most generally used for the protozoa found on the mucous surfaces of man. In this medium all of the amoebae and mucous surface flagellates found in man, with the exception of Giardia lamblia, will survive and multiply and the individual characteristics are well preserved. The preparation of this medium follows:

##### Eggs.

Sterile Ringer's solution (Autoclaved at 15 lbs. pressure for 20 minutes and then allow to cool). Prepared by mixing the following:

Sodium chloride. . . . .	8.0 grams
Potassium chloride . . . . .	0.2 grams
Calcium chloride . . . . .	0.2 grams
Distilled water. . . . .	1000 cc.

Sterile horse or human serum. (Must not contain tricresol or other preservatives.)

Emulsify four whole eggs in 50 cc. of the sterile Ringer's solution. Place 4 cc. of this mixture into each test tube and sterilize in an autoclave as follows: Turn the steam into the outer chamber of the autoclave until the jacket is hot, then place the tubes in a slanting position in the sterilizing chamber, close the door and be sure that the vacuum exhaust is also closed. Turn the steam into both chambers of the autoclave and open the outside exhaust valve. At the first appearance of steam from this valve close it and allow the pressure to climb to 15 lbs., at which time shut off the steam and (caution) allow the pressure to decline to 0 of its own accord before removing the media. Repeat this procedure on three successive days, storing the media at room temperature



between sterilizations. Prior to use, 4 cc. of the liquid portion of the medium, consisting of sterile horse serum one part and Ringer's solution eight parts, are added.

St. John's Wheat Broth Medium. This medium is of differential value in the cultivation of amoebae in that only E. histolytica can maintain growth in it. However, many strains of E. histolytica cannot be cultivated in it. Directions for preparation and use of this medium may be found in Laboratory Methods of the United States Army 4th Edition.\*

#### CULTURE TECHNIQUE.

Protozoa of this type are quite sensitive to changes in the bacterial flora in vitro; they die quickly in the presence of certain bacteria. Therefore, sterile precautions in so far as is possible, should always be used in this technique,

Routine Cultures. Using an applicator or wire loop inoculate a portion of the specimen about the size of a pea, consisting of fresh mucus, mucus and blood, or concentrated cysts, into the liquid portion of the medium and thoroughly emulsify it there. Incubate at 37°C. and examine at the end of 24 and 48 hours. Flagellates growing in the medium will be found throughout the liquid portion while amoebae will be found in the very bottom portion of the liquid fraction. The specimen from the culture to be examined for amoebae is obtained by introducing a clean sterile 1 cc. pipette, with the index finger held tightly over the upper end, into the culture so that the tip is at the bottom portion of the liquid medium, then gently release the finger pressure allowing only 1/10 cc. of the material in contact with the bottom to run into the pipette. Resume the finger pressure on the upper end of the pipette and then withdraw it from the culture. The material in the pipette can then be used to transfer the culture, make fresh wet unstained or stained preparations. If it is desired to carry on the positive cultures they should be routinely transferred every 48 hours. However, the survival of these organisms in culture depends a great deal upon the bacterial flora present. One in which Escherichia coli predominates is usually favorable and one in which spore bearers, Pseudomonas aeruginosa or Proteus vulgaris predominate is usually unfavorable. In the latter case transfer should be made at 24 hour periods.

Cultures for amoebae that are not positive at the end of 48 hours should be transferred and further examined as follows: Allow the culture to remain in the incubator for 2 hours, then without unduly disturbing it, remove all but 0.5 cc. of the supernatant fluid by means of a sterile pipette equipped with a rubber nipple. Wash the slant with the remaining fluid and then transfer it to new medium. The resulting culture should then be re-examined at 24 and 48 hour intervals before calling it negative,

#### LABORATORY ANIMALS.

Laboratory animals are of no value as a diagnostic procedure in this group. However, in case of E. histolytica, the virulence of this organism can be tested by injecting cultures or freshly isolated trophozoites into the rectum of a young kitten by means of small catheter and syringe. An acute fatal dysentery is usually produced.

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\* see bibliography

## SPECIAL METHODS USED IN THE DIAGNOSIS OF THE AMOEBAE

In the preceding pages technics were described to enable the technician to familiarize himself with laboratory methods of handling intestinal protozoa. Since Endamoeba histolytica is the intestinal protozoan of most interest to the medical soldier, special methods and aids for confirming diagnosis of infection with this parasite are outlined below.

Fresh, Unstained, Wet Mount. The fresh, unstained, wet mount prepared as outlined under general methods is examined systematically covering the slide without repeating the microscopic fields. Amoebae in the live state have a particular refractive, translucent, granular character which easily differentiates them from other objects in the specimen. This property can be exaggerated and will greatly aid in picking out the amoebae when the slide is being rapidly examined if the high power objective of the microscope is focused slightly above the objects in the preparation. If the observer is well trained, amoebae may be recognized under the low power objective and then a switch made to the high power objective to determine the detailed characteristics. If the outline below is followed in studying amoebae it will be found to be of great aid in species determination.

1. Size and color.

2. Differentiation of ectoplasm and endoplasm.

3. Granularity of endoplasm and presence of cell inclusions.

4. Nucleus - visibility, location when in motion, size.

5. Motility, type (active  
(sluggish  
(progressive or non-progressive

6. Pseudopodia (single (clear  
(or (or  
(multiple (granular

7. Flowing of endoplasm into pseudopod (slow  
(or  
(explosive

8. Presence or absence of (red blood cells in endoplasm  
(bacteria in endoplasm

9. Presence of (contractile vacuoles  
(food vacuoles

10. Presence and characteristics of chromatoid bodies.

11. Cultural characteristics (growth on R. M. S.  
(and St. John's Medium  
(on successive transfer



### Fresh, Iodine-stained, Wet Mounts.

1. Nucleus - size, shape, distribution of chromatin and size and uniformity of granules, location of karyosome.
2. Presence of glycogen bodies.
3. Cysts, if present - character of, size, number of nuclei and type, visibility, glycogen bodies.

Smears Stained with Iron-hematoxylin. Staining by hematoxylin methods should be made of all amoebae where species cannot be recognized readily in the fresh or iodine-stained preparations.

Cultures. Cultures properly made will increase the number of positive findings and allow further study in determining the true species of an amoeba.

Concentration of Cysts. Concentration methods for cysts should be done routinely on all fecal specimens that are examined.

Complement-Fixation. At the present time the complement-fixation test is still in the experimental stage in its development. It may become of practical diagnostic significance when a true E. histolytica antigen can be developed.

Charcot-Leyden Crystals. Charcot-Leyden crystals are indication of chronic inflammation and are often found in the feces.

Differentiation of Amoebic and Bacillary Dysentery. Differentiation of amoebic and bacillary dysentery can presumptively be done by the general character of the fecal exudate. However, one may complicate the other and amoebic dysentery in the presence of severe bacterial secondary invasion of the ulcers may have many of the characters of a bacillary dysentery. Therefore, every effort should be exerted to demonstrate the causative organism in each case. The main differential points are shown in the following table.

DIFFERENTIAL DIAGNOSIS TABLE \* ON THE FECAL EXUDATESIN BACILLARY AND AMOEBIC DYSENTERY

Exudate	Bacillary Dysentery	Amoebic Dysentery
Blood	Varying amounts	Small amounts to actual hemorrhage.
Polymorpho- neutrophiles	About 90% of exudate. Many show nuclear degen- eration (ringing). Cytoplasm frequently con- tains fat.	Few. Cytoplasm of some of those present shows degenerative changes and in such the nuclei may appear pyknotic.
Endothelial macrophages	Present in varying numbers. Actively phagocytic, frequently contain erythrocytes and leucocytes. Under- go toxic degeneration; "ghost cells".	Not seen except in cases also having bacterial dysentery.
Plasma cells	Present, relatively more abundant early.	Present in small numbers
Pyknotic bodies	Proportionately insignif- icant, but are found.	Constitute about 80% of cellular elements.
<u>E. histolytica</u> trophozoites	Absent unless the two diseases are both present.	Present and must be found to make diagnosis.
Amount of exudate, actual hemorrhage excluded.	Massive, a large part of the stool.	Small.
Bacterial content	Low.	Very high, usually.

\* see bibliography



Confusing Objects. Animal Tissue Cells, Tissue cells derived from the host, or ingested as food may at first glance appear as amoebae in the stools, but careful examination of them will easily establish their true nature. Macrophages may be found containing phagocytized red blood cells, but examination reveals their typical nuclear structure and amoeboid movement is not observed. Epithelial cells are pale in color and have nuclear characteristics that easily differentiate them.

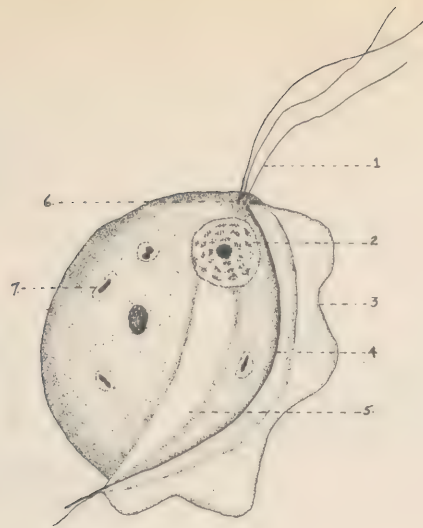
Vegetable Cells. Vegetable cells such as starch granules, pollen granules, yeast cells or other cells of this type have a certain definiteness of outline and structure that should lead to no confusion. However, yeast cells, such as Blastocystis hominis, may be confused with cysts of amoebae. The presence of budding forms and their particular structure should cause no difficulty in differentiating them.

#### Bibliography

Callender, G. R., The Cytological Diagnosis of Dysenteric Conditions and its Application in the Military Service, June 1925,

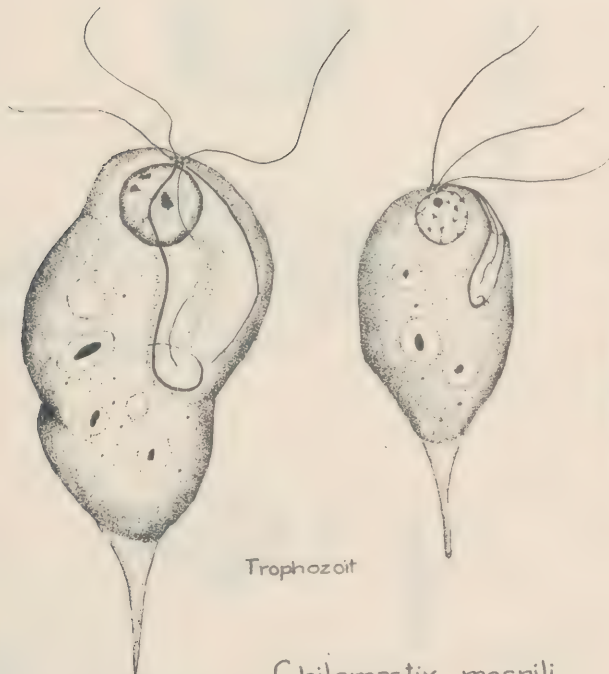




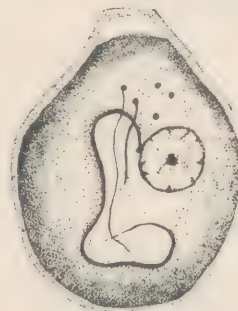


Trichomonas hominis (Davaine 1860)

- 1 Flagella.
- 2 Nucleus.
- 3 Undulating Membrane.
- 4 Basal fibre.
- 5 Axostyle.
- 6 Blepharoplast.
- 7 Food Vacuole.



Trophozoit

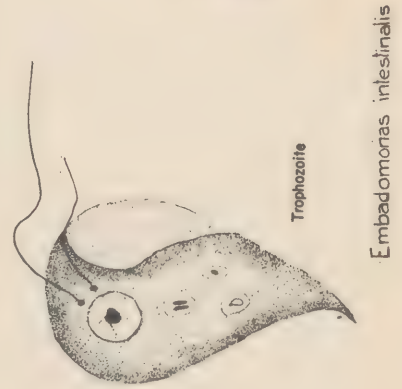
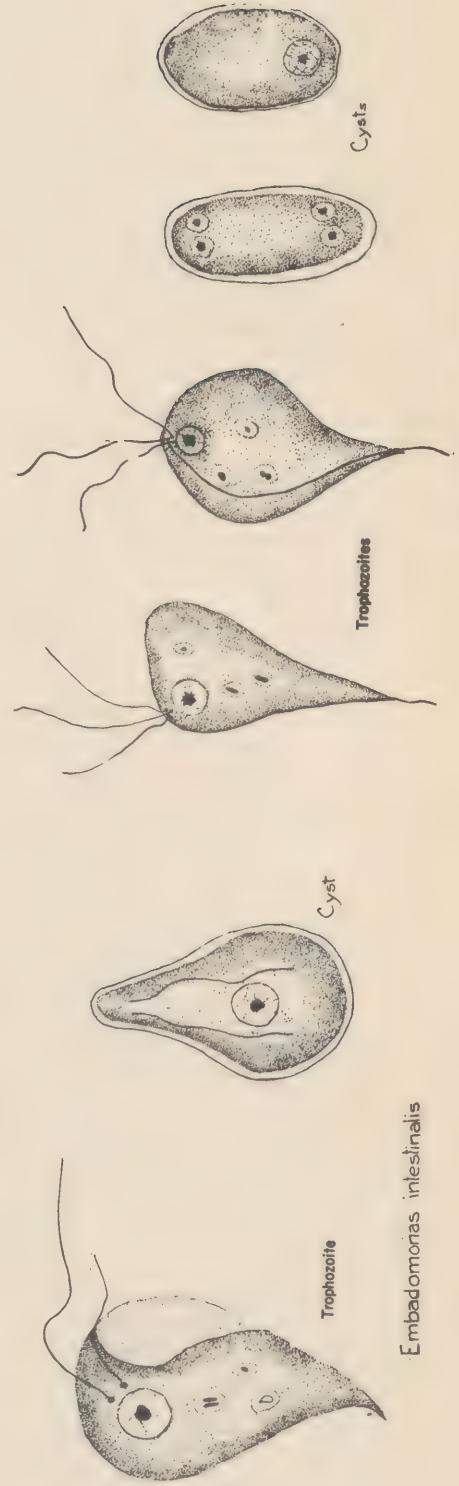
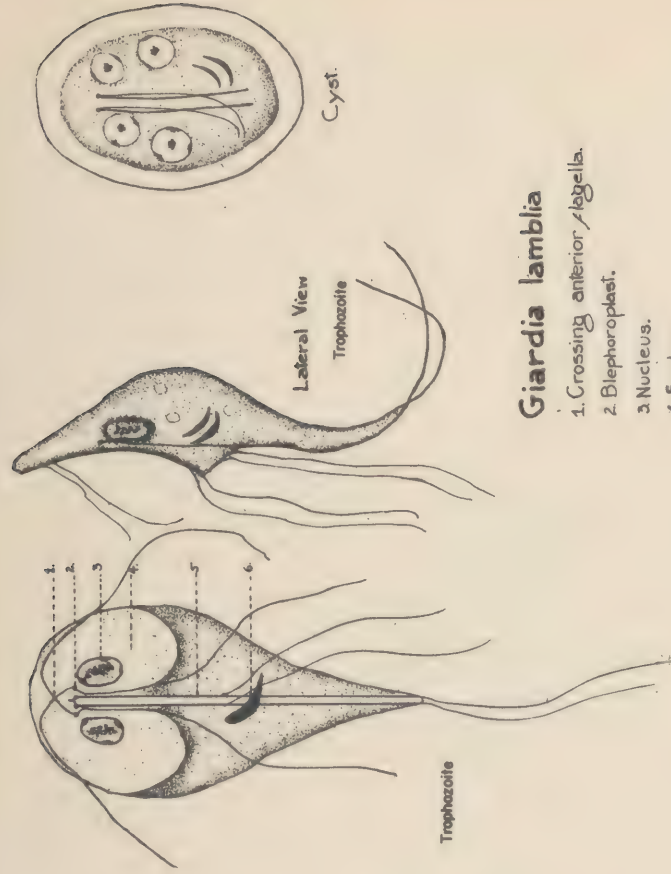
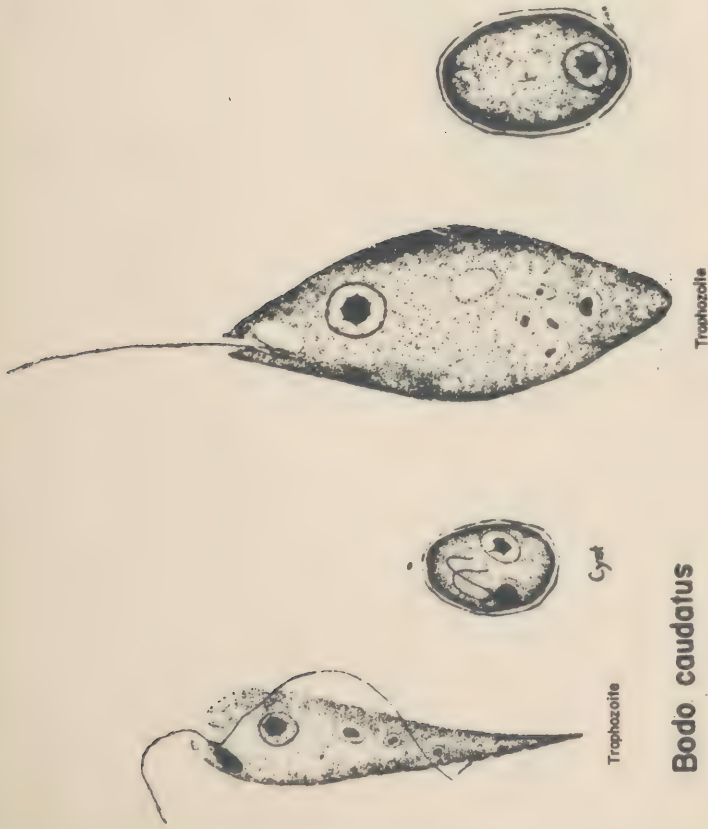


Cyst

Chilomastix mesnili













1. Peristome.
2. Cytostome.
3. Cytopharynx.
4. Food Vacuole.
5. Contractile Vacuole.
6. Micronucleus.
7. Macronucleus.
8. Cytopyge.
9. Cilia.

*Balantidium coli*





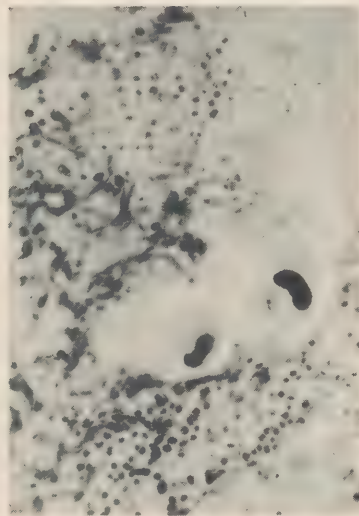


Life Cycle of *Eimeria perorans*  
in the intestinal epithelium of a  
Rabbit

- 1-7. Schizogony
- 8-13. Macrogametogony
- 14. Oocyst
- 15-19. Microgametogony



*Balantidium coli*  
in gut wall



*Balantidium coli*  
trophozoite





## THE BLOOD AND TISSUE FLAGELLATES

Morphology.- The flagellates found in the blood and tissues of man belong to the genus Leishmania and the genus Trypanosoma. The organisms in the flagellate group undergo changes in form during certain stages of their life cycles. These stages are generally referred to by the name designated for the form taken by the organism as illustrated by the following chart. Note that the genus Trypanosoma may assume any of the forms. The genus Leishmania has only two forms.

(Classification chart here)

## THE LEISHMANIAS

I. General.- There are three usually recognized species of Leishmania infecting man, although under the microscope the organisms are identical. The results of animal inoculation have been confusing regarding species differentiation. The diseases produced in man, however, are distinct, there being three types of lesions, visceral, cutaneous and muco-cutaneous.

II. Leishmania donovani (visceral form). - Infection with this parasite is called kala-azar, i.e. "death fever", and is fairly common in the countries bordering the Mediterranean, India, China and South America. (The Mediterranean type affects children chiefly, but many adults have the infection and it is not uncommon in aged people.) The disease is chronic and is characterized by anemia, loss of weight and marked enlargement of the spleen. The parasites are found in the endothelial cells of the liver, spleen, lymph nodes and occasionally in the white blood cells circulating in the blood. It is generally agreed that the parasites are transmitted to man thru the bite of an infected fly of the genus Phlebotomus (see section on entomology), but there is still no definite proof that any insect is the transmitting agent.

1. Morphology.- In the tissues this species is a very small (1 to 3u) round or oval body, with a sharp outline and poorly staining cytoplasm. With Wright's stain the cytoplasm stains a pale blue, the nucleus appearing as several bright red granules. The para-basal body stains deep purple and is the only other definite structure clearly visible in the parasite in man (see fig. ).

(Insert figure)

2. Diagnostic Methods.- The laboratory methods of diagnosis are, in order of reliability, culture of material obtained from spleen or liver puncture, direct examination of this material, the aldehyde test, and the direct examination of the blood. Complement-fixation and the precipitin tests have been used by certain workers with some degree of success.

(a) Culture.-- The NNN (triple N) medium is ordinarily successful. This medium is made of

Agar	14 gm.
Sodium Chloride	6 gm.
Distilled Water	900 cc,

Mix and dissolve by means of heat, then tube in 6 cc. amounts and autoclave for thirty minutes under 20 pounds of steam pressure. Remove the tubes and cool to 48°C. Then under aseptic conditions add 2 cc. of sterile defibrinated rabbit's blood to each tube, mix well and slant. Slanted tubes should be placed in the ice box to cool and harden so they will have the maximum amount of water of condensation. When cool the cotton plugs of each tube should be covered with a rubber cap to prevent evaporation of the water. The tubes should be tested for sterility by incubation for twenty-four hours before they are used, because trypanosomes will not grow in the presence of bacterial contamination. The material suspected of containing Leishmania is placed at the bottom of the slants in the water of condensation and incubated at 22 to 25°C. for 3 to 14 days.

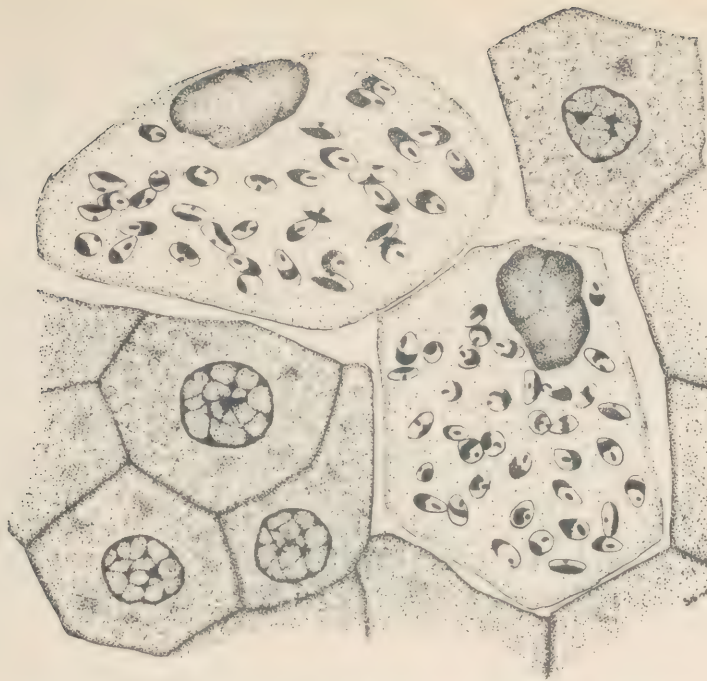
(b) Spleen and Liver Puncture.-- This is a procedure that should only be done by a competent medical officer due to the danger of tearing the organ and causing uncontrollable hemorrhage. The material obtained is spread on a slide in as thin a layer as possible and stained with Wright's or Giemsa's stain. Examination is microscopic.

(c) Aldehyde Test.-- To 1 cc. of clear serum obtained from the blood of a suspected case add 2 drops of 100% formalin (i.e. 40% formaldehyde solution). If the infection is kala-azar the serum will jelly within a few seconds to thirty minutes. While this test may also be positive in malaria, tuberculosis and leprosy, it is nevertheless of value.

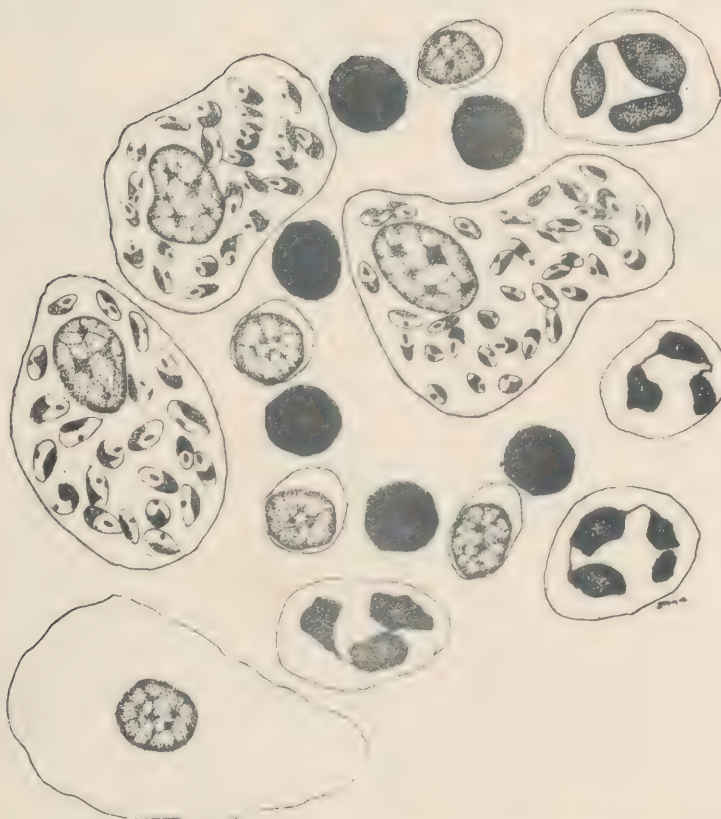
(d) Blood.-- Leishmania are rarely recovered in the circulating blood. When present they are found in monuclear and polymorphonuclear leucocytes.

III. Leishmania tropica (cutaneous form).-- Infection with this parasite, which is common in the Mediterranean area and in Africa, is known as oriental sore, Delhi boil and buas. The organisms are found in nodules just under the skin and in the margins of ulcers, and may be obtained by puncture and aspiration or by scraping the margins of the ulcers. Material so obtained is spread on a slide and stained and examined as for L. donovani. Since the organisms are very scarce, much patience must be used in the search. The parasite may be cultivated in the NNN medium, but usually there will be too many bacteria present.





*Leishmania donovani* in the Kupfer Cells of the Liver



*Leishmania tropica* in Smear from Cutaneous Lesion





IV. Leishmania brasiliensis (muco-cutaneous form).-- This organism produces the muco-cutaneous leishmaniasis or espundia of Central and South America. Its morphology is the same as the other genera described above. The above mentioned NNN medium is again used in culturing this parasite. The method of transmission is unknown although it is believed to be by the sandfly Phlebotomus intermedius which is common in regions where the disease is found.

#### THE TRYPANOSOMES

I. General.-- Trypanosomes are protozoa of a somewhat elongate shape, and tapering at both ends. Along the convex margin is an undulating membrane, to which is attached the flagellum. The flagellum arises from a small granule, the blepharoplast near the back end of the organism. It then passes along the free margin of the undulating membrane and may emerge at the anterior end of the organism as a free flagellum of variable length. Just posterior to the blepharoplast there is usually a dark, shining, round or rod-like body known as the parabasal body. The nucleus of the organism is round or oval and centrally located. The cytoplasm is clear but may contain granules which stain red with Giemsa's stain. Figure \_\_\_\_\_ shows the internal structure of a trypanosome,

(Insert figure)

The trypanosomes of medical interest are:

1. Trypanosome gambiense and T. rhodesiense, the causative organisms of African sleeping sickness. For discussion of these the interested worker is referred to standard texts # in this field.
2. Trypanosoma cruzi, the causative organism of Chagas' disease. This parasite is discussed in detail below.
3. Trypanosoma lewisi, a trypanosome of rats, which though not important medically, is of interest since it may be confused with T. cruzi.

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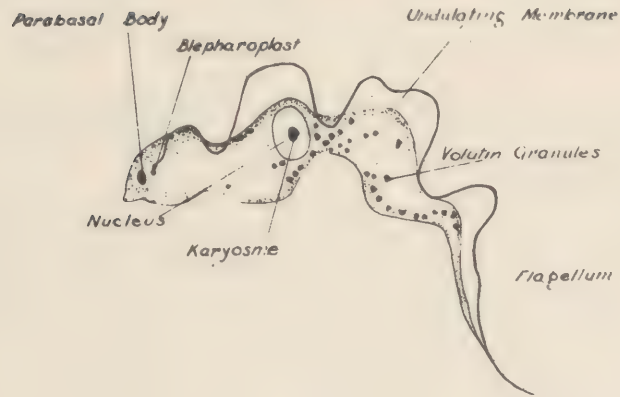
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# TRYPANOSOMES OF MEDICAL IMPORTANCE

Species	Length	Definite Host	Intermedia Host	Geographical Distribution	Susceptible Animals	Culture	Disease
<u>T. gambiense</u>	15-30u	Man, domestic animals	Tsetse fly <u>Glossina palpalis</u>	Tropical Africa, but mostly Western and Central.	All Laboratorial Animals except Monkey	NNN Plus Glucose	Gambian Sleeping Sickness
<u>T. rhodesiense</u>	12-35u	Man, big game animals- Antelope	Tsetse fly <u>G. morsitans</u>	Eastern Africa " Rhodesia, etc.	"	"	Rhodesian Sleeping Sickness
<u>T. cruzi</u>	20u	Man, Cat, Armadillo, Opossum, wood rats, bats	Kissing Bug <u>Panstrongylus megistus</u>	South and Central America Organism found in reservoir hosts in U. S.	Guinea Pigs, White Rats, Monkey	NNN	Chagas' Disease



### Diagram of a Trypanosome



### Forms Found in Life Cycle of Trypanosomes

- |                |                 |
|----------------|-----------------|
| 1. Leishmania. | 3. Crithidia.   |
| 2. Leptomonas. | 4. Trypanosoma. |





Rats are used in the laboratory in animal inoculation methods of diagnosis of Chagas' disease, and since T. lewisi and T. cruzi are very similar in appearance, the rats used in diagnosis must be free from T. lewisi.

(put chart here)

II. Trypanosoma cruzi (Chagas' disease).-- This is a fairly common trypanosome infecting man in tropical and subtropical South and Central America. Although this is primarily a childhood disease, it is sometimes found in adults.

1. Life Cycle.-- In man T. cruzi as seen in the peripheral blood is a trypanosome about 20 u in length. Its body tends to be shaped like the letter C and the posterior end is sharply pointed. The parabasal body is very conspicuous and oval in shape. There is a free flagellum. The nucleus is oval in shape and centrally located. There may be some variations in the width of the body, as the young forms are narrower than the old. After a variable period in the blood stream, the mature trypanosome invades the muscle fibers or other tissue cells, assumes the leishmania form and multiplies by splitting lengthwise. This multiplication of the leishmania stage continues until the cell ruptures from so many parasites. Each then develops a short flagellum, the body lengthens, the parabasal body moves posteriorly and the young trypanosome now returns to the blood stream or invades other tissue cells. There is considerable local tissue damage and destruction due to the invasion of tissue cells by this parasite. In the triatomid bug (see section on entomology) about six hours after the bug has fed upon an infected individual the ingested trypanosomes round up and assume the leishmania form. These forms multiply rapidly as they pass along the intestinal tract. In the hind-gut they again develop flagella and in about twenty-five to thirty hours become crithidial forms (see diagram). These forms multiply in the hind-gut and are found in greatest numbers near its junction with the kidney tubules. The crithidial forms now develop into trypanosome forms which are infective for man. When the bug feeds again, it deposits a small drop of fluid from the rectum upon the skin of the individual. This fluid excrement may contain great numbers of trypanosomes which are capable of passing through intact mucous membrane or small skin abrasions into the blood stream, thus infecting the individual. Persons may inoculate themselves by accidentally rubbing the deposited feces into the feeding-site,

## 2. Diagnostic Methods.--

(a) Collection of the Specimen.-- The most favorable time to find these organisms in the suspected infection is during the periods of fever. In most cases of trypanosomiasis there are usually very few organisms present in the blood stream. It is necessary therefore to use concentration methods as well as thick blood films. A suitable concentration method follows: Using sterile technique, 10 cc. of blood are withdrawn from a vein and immediately mixed with 40 cc. of sterile, warm (37°C.) distilled water in a 50 cc. sterile centrifuge tube. The tube is shaken and as soon as hemolysis is complete it is centrifuged at approximately 2500 R.P.M. for five minutes. The supernatant fluid is decanted and the trypanosomes present in the same will be found in the residue, which can then be examined microscopically. If the preparation has been kept sterile it may be used to inoculate culture media.

Lymph-gland puncture is the method most apt to give positive results. One of the enlarged lymph glands is punctured aseptically with a Luer syringe equipped with a 19 gauge needle and some of the gland juice is aspirated. This aspirated material may then be examined under the microscope, cultured, or inoculated into a susceptible animal.

(b) Slide Preparations.— Fresh, unstained, wet mounts and thin and thick stained films can be prepared as outlined in the section on malaria. Parasites are not apt to be numerous.

(c) Culture.— Concentrated, laked blood; aspirated contents of lymph glands; spinal fluid; or biopsy tissue from a suspected case of trypanosomiasis, when inoculated into a suitable medium may yield cultures positive for trypanosomes.

One of the best media upon which to culture T. cruzi is the NNN medium described in the section under Leishmania.

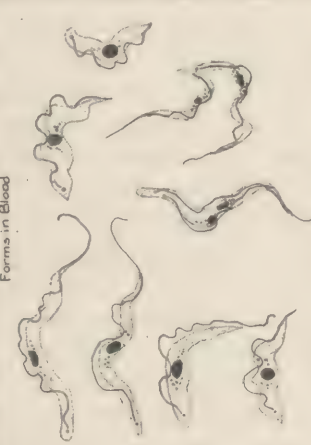



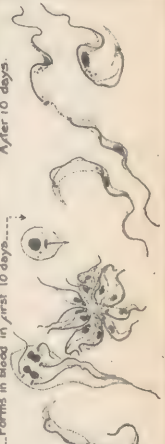

(d) Animal Inoculation.— In diagnosis, by animal inoculation either the blood or an emulsified portion of an infected lymph node may be injected intraperitoneally into a guinea pig, white rat or monkey—preferably guinea pig.

#### REFERENCES

- Craig, F. G., and Faust, C. F., 1940, Clinical Parasitology, Philadelphia, 771 pp.  
 Manson-Bahr, P. H., 1940, Manson's Tropical Diseases, Baltimore, 1083 pp.  
 Thomson, J. G., and Robertson, A., 1929, Protozoology, New York, 376 pp.



# DIFFERENTIAL CHART OF TRYPANOSOMES OF MEDICAL IMPORTANCE

Species	Geographic Distribution	Length	Type	Basion or Nucleus Size and Shape	Parabasal Body Shape	Drawings from Blood Smears and Tissue	Dividing Forms in Peripheral Blood	Leishman Forms in Tissue or Blood	Vertebrate Host	Invertebrate Host (Vectors)	Susceptible Laboratory Animals	Culture	Disease Produced
<i>T. gambiense</i>	Tropical Africa	15 $\mu$ to 40 $\mu$	Bilymorphic (forms without and with flagella in blood stream)	Middle $\frac{1}{2}$	Small inconspicuous	<p>Forms in Blood</p> 	Yes	No	Man and probably Antelope	Tsetse Fly <i>G. morsitans</i> <i>G. palpalis</i> <i>G. brevipalpis</i> <i>G. pallidipes</i> <i>G. tachinoids</i> (bite injective after 20-30 day cyclic development)	All except monkeys	Culture Media NNN plus glucose 32°C. Ponselle's medium at 25°C Cultivation difficult.	African sleeping sickness of Man.
<i>T. rhodiense</i> (probably only a virulent strain of <i>T. gambiense</i> )	East Africa (Rhodesia, Nyasaland, Tanganyika, Mozambique)	18 $\mu$ to 35 $\mu$	Polymorphic	Middle $\frac{1}{2}$ and sometimes posterior may be behind parabasal body	Small inconspicuous	<p>Parasites same as <i>T. gambiense</i> except in laboratory animals in which posterior nuclear forms develop.</p> 	Yes	No	Man and probably Antelope	Same as for <i>T. gambiense</i>	All except monkeys	Same as for <i>T. gambiense</i>	African sleeping sickness of Man. Virulent form.
<i>T. cruzi</i>	South America and probably Central America	16 $\mu$ to 24 $\mu$	Mono-morphic	Middle $\frac{1}{2}$	Conspicuous Large and Oval	<p>Forms in blood</p>  <p>Forms in Tissue</p> 	Never	In Tissue	Man Armadillo probably Cat Opossum	Kissing Bug <i>T. megistoma</i> , <i>T. infestans</i> , <i>T. sordida</i> , <i>Rhodnius prolixus</i> , <i>Cimex lectularius</i> , fecal excrement injective after 10-20 day cyclic development	Guinea pigs Rats Mice Rabbits Dogs Cats Monkeys	Culture Media NNN 25°C	Chagas disease South American Trypanosomiasis of Man.
<i>T. lewisi</i>	Cosmopolitan	20 $\mu$ to 30 $\mu$	Mono-morphic	Anterior $\frac{1}{3}$	Large and Rod shaped	<p>Forms in blood in first 10 days</p>  <p>After 10 days</p> 	Yes	In Blood	Rats	Fleas, (C. canis, E. lexis irritans, etc.)	White Rats Mice with difficulty	Culture Media NNN 25°C	Trypanosomiasis of Rats Non-pathogenic for Man.





## THE GENUS PLASMODIUM (MALARIA)

The parasites of this genus are single celled, pigmented, amoeboid organisms, living within the red blood corpuscles of man or some other vertebrate. The asexual cycle is confined entirely to the red blood cells of man or animals, whereas the sexual cycle is found in the mosquito. The figure following shows diagrammatically the life cycle of the malaria parasite in man and the mosquito.

I. PLASMODIUM VIVAX.

1. LIFE CYCLE IN MAN. - The sporozoites, which constitute the infective stage for man, are injected into the body during the bite of the infected female anopheline mosquito. After reaching the blood stream these elongated forms (sporozoites) parasitize red blood cells, round up, and assume the typical signet-ring form. These ring forms are about  $1/3$  to  $1/4$  the diameter of the infected red cell and when stained by Wright's or Giemsa's method, the chromatin stains red and the cytoplasm blue. In about six hours, the parasite increases in size by one-third and shows marked amoeboid movement within the infected cell. The infected red cell has now enlarged until its diameter is from 10 u to 12 u and its color is paler than the uninfected red cells. If the infected red cells are properly stained, fine pink dots or stippling (Schuffner's dots) are usually present in the cytoplasm, and minute granules of light brown pigment are seen near the chromatin of the parasite. The growth of the parasite is fairly rapid and by the end of thirty-six hours it fills two-thirds of the enlarged stippled, parasitized red cell. After about forty hours, the parasite fills the cell and its chromatin usually divides into 12 to 16 segments. Small yellowish brown granules of pigment are now scattered throughout the body of the parasite. The pigment then clumps toward the center of the parasite whose cytoplasm then divides into equal sections, one about each segment of the chromatin. The small bodies resulting from the division of the mature parasite are called "merozoites". At the end of forty-eight hours the merozoites rupture the red cell membrane, (at which time the patient's chill begins), and then each liberated merozoite seeks out and parasitizes a new red blood cell, to repeat the cycle. The pigment is liberated into the blood stream where it is destroyed. At any given time, practically all of the stages in the life cycle can be demonstrated in the blood stream, although one stage always predominates. The probable reason for this is that infections in man are due to the bite of more than one infected mosquito, or that in a single bite, some of the sporozoites instead of being inoculated intravenously are inoculated subcutaneously, thus delaying the start of their life cycle. P. vivax requires 48 hours for completion of its asexual cycle.

The gametocytes (which will become sexual forms when taken up by the mosquito) develop from certain undifferentiated merozoites. The stimulus for their formation is unknown, but it is probably a response to developing protection on the part of the host. Four days are required for the development of the ring forms into mature gametocytes, and these must be from seven to ten days old before they are infectious for the mosquito. When fully grown the gametocytes are rounded in form and have fairly uniform cytoplasm. They appear in



the blood cells within about seven days after the initial fever. Their life in the blood stream is about ten to twenty days. They are incapable of reproducing themselves or of starting the asexual cycle in man without first undergoing changes in the female anopheline mosquito.

(1) The male gametocyte (microgametocyte) is 7 u to 8 u in diameter and occupies an enlarged red cell without completely filling it. Schuffner's dots may be present in the margin of the red cell. The cytoplasm of the parasite stains faintly blue-grey and contains numerous granules of brown pigment. The chromatin stains red and is scattered over a fairly wide area, sometimes in the shape of a band, near the center or edge of the parasite.

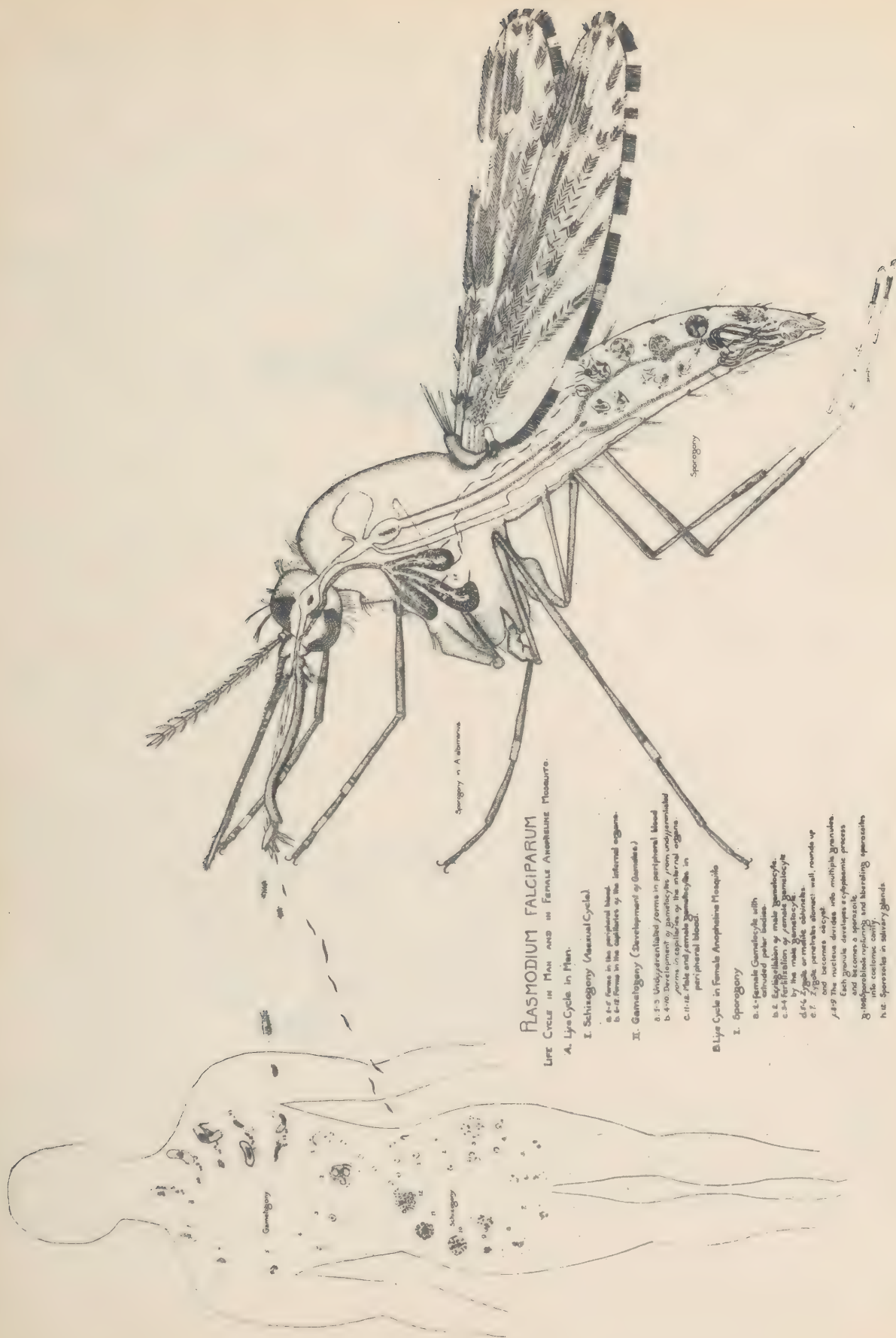
(2) The female gametocyte (macrogametocyte) is larger, 8 u to 10 u, fills the parasitized cell more completely and its cytoplasm stains a darker blue than the male gametocyte. The chromatin of the female gametocyte is in one compact clump, usually near the edge of the parasite, and the pigment is in coarse brown compact granules. There are usually from 3 to 6 female gametocytes for every male gametocyte in the peripheral blood.

2. LIFE IN THE FEMALE ANOPHELINE MOSQUITO. - The gametocytes are drawn into the mosquito's stomach along with its blood meal. Within twenty minutes, the male gametocyte develops eight to ten flagellar-like processes, which soon exhibit violent lashing motion. These processes soon separate from the original cell body and are then called "microgametes". While this process has been going on in the male gametocyte, the female gametocyte extrudes two bodies, each containing one-half of the chromatin. These are now ready for fertilization and are called "macrogametes". The microgamete penetrates the cell wall of the macrogamete, the chromatin of each fuses and the parasite now becomes a motile organism called an ookinete. The ookinete now penetrates through the wall of the mosquito's stomach, rounds up, becomes immotile, and is then called an "oocyst". The chromatin of the oocyst undergoes multiple divisions, until there may be as many as 10,000 granules, which are grouped together in small clumps. The oocyst now contains from ten to twenty groups of these chromatin granules and is known as a "sporoblast". Each chromatin granule now develops a cigar shaped cytoplasmic process and becomes a sporozoite. The sac containing the sporozoites is called a "sporocyst". The sporocyst ruptures into the body cavity, liberating the motile sporozoites, which soon get into the mosquito's salivary glands. This process in the female anopheline mosquito requires about seven to twelve days for its completion, if the temperature is about 20°C. and the air is about 70% saturated with moisture. Then the mosquito is ready to infect a new person. The infection apparently does not harm the mosquito.

## II. PLASMODIUM MALARIAE.

This parasite causes quartan malarial fever and it is the rarest of the three common species in man. Except for infected travelers, it is probably confined to the tropical or subtropical portions of the world. In subtropical regions, the greatest number of new cases of this infection occur during the fall months. The symptoms of this infection are much more severe than would be expected from the number of the parasites infecting the red cells. In this infection, all stages are usually present in the blood stream at one time, although one stage always predominates.





# **PLASMODIUM FALCIPARUM** LIFE CYCLE IN MAN AND IN FEMALE ANOPHELINE MOSQUITO.

## **A. Life Cycle in Man.**

### **I. Schizogony (Asexual Cycle).**

- a. 1-2 Forms in the peripheral blood.
- b. 4-12 Forms in the capillaries of the internal organs.

### **II. Gametogony (Development of Gametes).**

- a. 1-3 Undifferentiated forms in peripheral blood.
- b. 4-10 Development of gametocytes from undifferentiated forms in capillaries of the internal organs.
- c. 11-12 Male and female gametocytes in peripheral blood.

## **B. Life Cycle in Female Anopheline Mosquito.**

### **I. Sporogony**

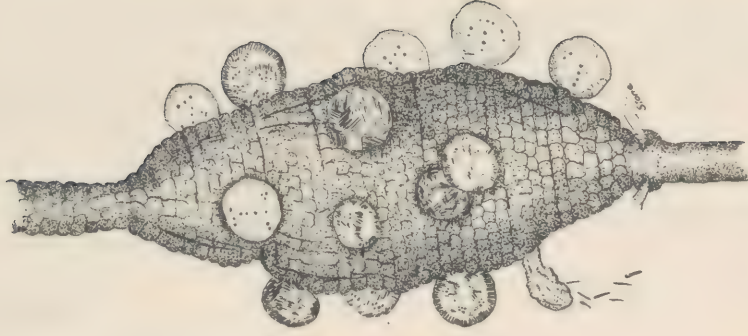
- a. 1-4 Female gametocytes with undivided polar bodies.
- b. 2 Excystation of male gametocytes.
- c. 2-4 Fertilization of female gametocytes by male gametocytes.
- d. 1-2 Trophozoites in the midgut.
- e. 1 Trophozoite penetrates abdominal wall, moults up and becomes oocyst.
- f. 2-9 The nucleus divides into multiple granules. Each granule develops a cytoplasmic process and migrates to the periphery of the oocyst.
- g. Trophozoite migrates into the coelomic cavity and liberating sporozoites.
- h. 12 Sporozoites in salivary glands.







Portion of Mosquito Salivary Gland Showing  
Injection with Malarial Sporozoites  
(oil immersion magnification)



Mosquito Stomach Showing Malarial Oocysts in  
Various Stages of Development  
(low power magnification)





1. LIFE CYCLE IN MAN. - The human phase of Plasmodium malariae is similar to that of P. vivax with the following exceptions: (1) P. malariae requires 72 hours to complete its asexual cycle. (2) During the first 6 hours of its growth it has the largest ring form of all the types of malaria, and when it is 24 to 48 hours old, it develops typical band forms. (3) Its segmenting forms only develop 8 to 12 merozoites. (4) The infected red cells are not enlarged. (5) Schuffner's dots are not present. (6) The pigment present is coarse and stains dark brown or black.

The gametocytes in Plasmodium malariae are similar to those in P. vivax, except that the organisms are smaller and the red cells are not enlarged, and do not contain Schuffner's dots. The time required for its gametocytes to develop is approximately seven days.

2. LIFE CYCLE IN THE FEMALE ANOPHELINE MOSQUITO. - The life cycle in the female anopheline mosquito is the same as for P. vivax, except that it takes about three times as long to complete the cycle.

### III. PLASMODIUM FALCIPARUM.

This is the organism of estivo-autumnal, subtertian, malignant tertian or pernicious malaria. Its distribution is similar to that of Plasmodium malariae, but it is second only to P. vivax in the frequency of its occurrence. It is the most severe of all the malarial fevers of man and requires early diagnosis and prompt treatment.

1. LIFE CYCLE IN MAN. - The asexual cycle is similar to that of P. vivax with the following exceptions: (1) The ring forms are smaller when they first parasitize a red blood cell. (2) There are more apt to be multiple infections of a red cell. Ring forms may show two dots of chromatin. (3) The rings may persist for about 24 hours in the peripheral circulation during which time they double or triple in size. (4) Ring forms, and mature gametocytes which are crescent shaped ordinarily are the only forms found in the peripheral blood. In severe, overwhelming infections by this parasite peculiar thread-like forms may occasionally be present in the peripheral blood. (5) Developing ameoboid forms and gametocytes are usually found only in the capillaries of the internal organs (spleen, liver, etc.). These organisms have a marked tendency to clump together and stick to the endothelial cells, thus bringing about capillary blockage. (6) Segmenting forms of P. falciparum develop 16 to 32 merozoites. (7) The parasitized red blood cells are not enlarged. (8) Schuffner's dots are not present in the parasitized cells, but large granules staining purplish red, called "Maurer's dots" or malignant stippling are present.

The gametocytes are crescent-shaped with rounded ends and they are greater in length than the diameter of the red cells which they have parasitized. The male gametocyte stains bluish-gray and has a central nucleus with diffusely scattered chromatin. The pigment present is in coarse grains and stains brownish-black. It is scattered between the grains of chromatin. The tips of the male crescent are usually more rounded than those of the female crescent. The female gametocyte (crescent) stains sky-blue and its nucleus is made up of a compact mass of chromatin and pigment.



2. LIFE CYCLE IN THE FEMALE ANOPHELINE MOSQUITO. The life cycle of P. falciparum in the female anopheline mosquito is essentially the same as that of P. vivax, except that  $1/3$  to  $1/2$  again as much time is needed to complete the cycle.

#### IV. PLASMODIUM OVALE.

This organism resembles P. malariae except that in stained preparations the ring forms show Schuffner's dots and the infected red blood cells are oval shaped. The red blood cells are larger than normal, but not so large as cells infected with P. vivax. This malarial infection is rare in the Americas but some cases have been recently reported. The following chart shows graphically the differential diagnostic points in the three genera commonly affecting man.

### LABORATORY EXAMINATION FOR MALARIA

#### I. PRECAUTIONS IN COLLECTING SPECIMENS.

1. The most favorable time to find malarial parasites in the blood in a clinical case of malaria is the period beginning twelve hours after the chill up to one hour before the next chill. Do not take specimens of blood for examination during a chill.

2. Suspected positive findings should be confirmed by the laboratory officer before being reported.

3. Quinine or other anti-malarial drugs used in treatment within four days before taking the sample make it very difficult to demonstrate the parasites except by the thick film method.

4. Repeat the examination as many times as necessary to prove or disprove the diagnosis.

5. Use only glass slides that are chemically clean and free of scratches, grease, or fogging.

#### II. PREPARATION OF SPECIMENS.

1. THIN BLOOD FILMS. - Thin films for staining are prepared by securing blood from the patient, and then making thin smears on a clean slide as if for a differential white blood cell count. The smear should be so thin that the cells are in a single layer and do not override one another. When the smear is too thick it is usually due to the use of too large a drop of the patient's blood or to the streaking slide being held at an angle of less than 35 degrees when the smear was made. The smear should not be blotted, but allowed to air dry rapidly without the use of heat. Do not blow the breath upon the smear to hasten drying, because it will lake the red blood cells. If smears are stained before they are dried, the cells will not be properly fixed, the stain will be precipitated, and the result will be unsatisfactory. The following method is recommended:



(1) Flood the thoroughly dried blood smear with the Wright's stain for one-half to one and one-half minutes.

(2) Dilute the stain on the slide with an equal volume of buffered distilled water pH 6.8 or enough of the water so that there is produced a metallic scum on top of the mixture. Allow this mixture to stand for three to five minutes.

(3) Thoroughly wash the stained blood film with neutral distilled water until it is light pink in color, then blot and allow to air dry.

2. THICK BLOOD FILMS. - Thick films for staining are made by securing a large drop of the patient's blood on a clean glass slide, then spreading the drop with the corner of another slide so that the blood covers an area about the size of a ten cent coin. The film is then allowed to air dry thoroughly at room temperature or in 37°C. incubator. If the film is thoroughly dried before it is stained, the blood cells will stay on the slide. Thick smears are best stained by the Giemsa method (see combined thick and thin film method). However, if this stain is not available, they may be stained with Wright's stain, using the following method:

(1) Immerse the thoroughly dried thick smear for 10 minutes in a solution made of:

Formalin..... 5 cc.  
Acetic Acid..... 1 cc.  
Distilled water q.s.ad..... 100 cc.

This solution fixes the parasites and white blood cells, but dissolves the red cells.

(2) Remove the smear and wash thoroughly in tap water in a Coplin jar following the distilled water.

(3) Allow slide to thoroughly dry and then stain by Wright's method as for a thin smear.

3. COMBINED THICK AND THIN STAINED BLOOD FILMS. - The thick stained smear and the combined thick and thin stained smears are prepared and stained by Giemsa's or Wright's method as outlined below. They are the methods of choice in searching for malarial parasites in carriers, clinical cases that have very few parasites in the peripheral blood and in malarial surveys. The appearance of the parasites in a stained thick preparation after the red cells have been laked out is different from that in stained thin preparations. They are quite typical, but the body of the parasite may be distorted in shape due to the destruction of the red blood cell. Therefore, where possible, the novice should not use this method until he or she has become thoroughly familiar with the malarial parasites and those confusing objects that are found in the thick stained smear. A convenient way of handling and staining large numbers of thick smears in a malarial survey is the one outlined by Barber and Komp of the United States Public Health Service. "In handling large numbers of thick smears it is convenient to carry out the technique in groups of 25 slides. With this in mind, the thick film is placed about one inch from one end of the slide and



the other end is used for labeling. The slides are assembled in groups, a cardboard 1/16 to 1/8 inch thick and 1 1/2 inches long, is inserted between the slides at the labelled ends and the whole fastened together by means of a stout rubber band. The entire block may now be stained and dried as a single unit. The combined thick and thin smears for staining are prepared by making a thick smear on one end of the slide and a thin smear starting one-half inch from the thick smear, and then streaking it towards the opposite end of the slide. Draw a line with a wax pencil between the two smears and they are now ready for staining. Proceed as for a thick smear, but, be careful to immerse only the thick smear in the acidulated formaldehyde solution. If the thin smear comes in contact with this solution, the red cells will be dissolved out and the smear will be useless. Failure to stain by Wright's method is usually due to insufficient lapse of time after diluting the stain with distilled water, or to contamination of the stain, or other reagents, or material, with acid. The precipitation of granules of stain on the blood film is either due to improper drying of blood films before starting the stain, introduction of water into the stock stain, or too much evaporation of the alcoholic stain before dilution. Red cells stained blue, except for the occasional cells showing polychromatophilia, are either overstained, (too much time allowed after diluting the stain) or have been insufficiently washed during the last stage of the staining process."

4. STAINS. - See the section on hematology.

III. Examination of Specimens.

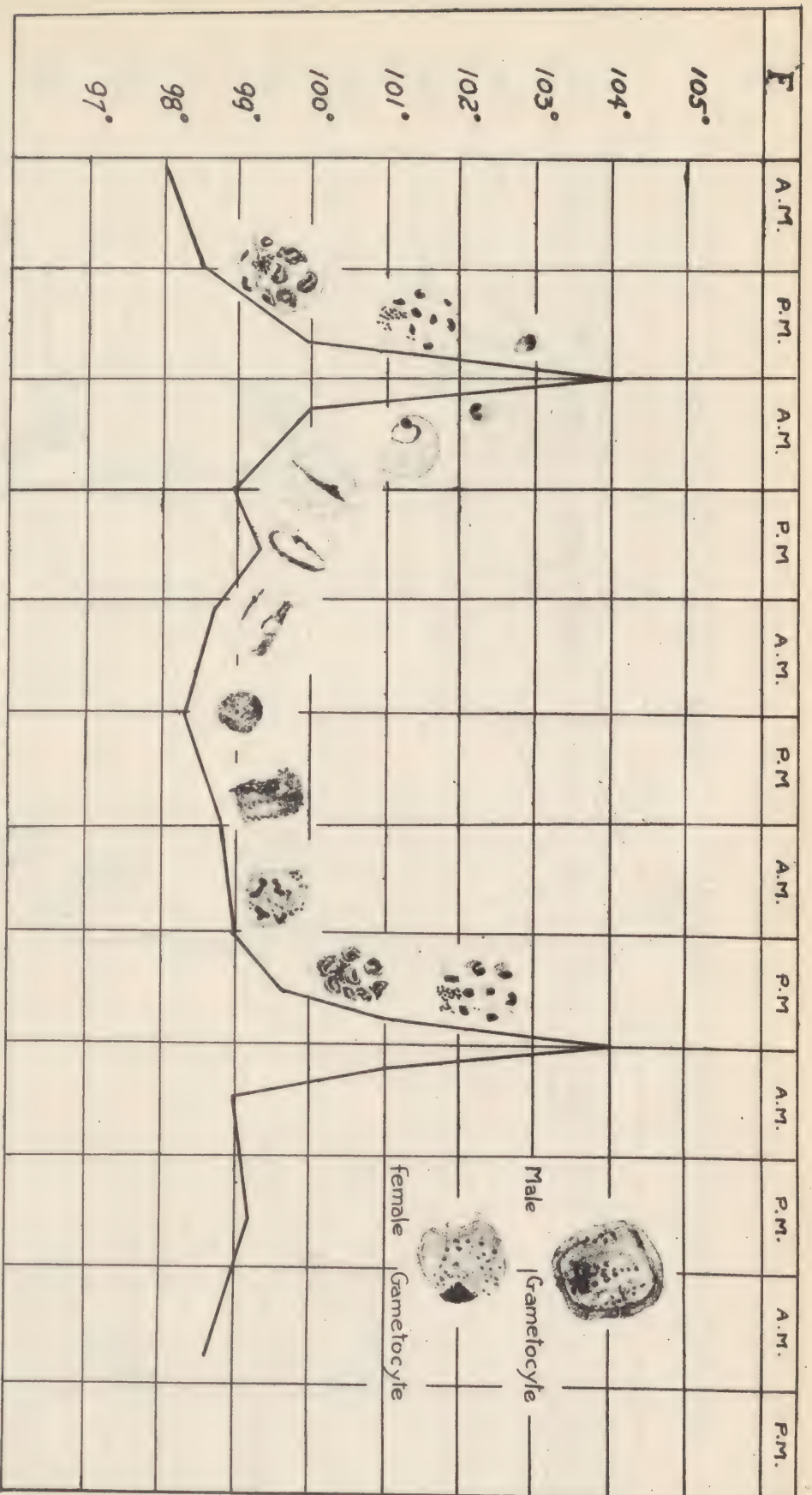
The laboratory diagnosis of malaria infection in man consists of finding and recognizing the malarial parasites, and their type, when present in the blood preparations made from suspected malarial fever patients. In examining preparations made for malaria the oil immersion objective should be used. Malarial parasites are best seen when the light coming through the substage is slightly reduced. The proper amount of light may be obtained by moving the slide until a blood platelet is centered in the field, then adjusting the substage so that the maximum definition of its morphological detail is obtained. In searching for the parasites the slide should be covered in an orderly manner moving back and forth over the smear so as not to repeat any field previously examined. Never make a diagnosis on the first parasite found, cover enough of the slide so that if two species of malarial parasites are present you will find them. If in doubt about any single abnormal parasite found, remember that where there is one malarial parasite there are bound to be more and careful search will usually reveal an easily recognizable form. When the technician has determined the presence of malarial parasites he should submit the slide to the laboratory officer for confirmation. No positive reports for malarial parasites should leave the laboratory except those slips signed by a responsible officer. If no officer is available and you are sure there are malaria parasites present report as a suspected positive. Save the slide for a final confirmation at a later date.



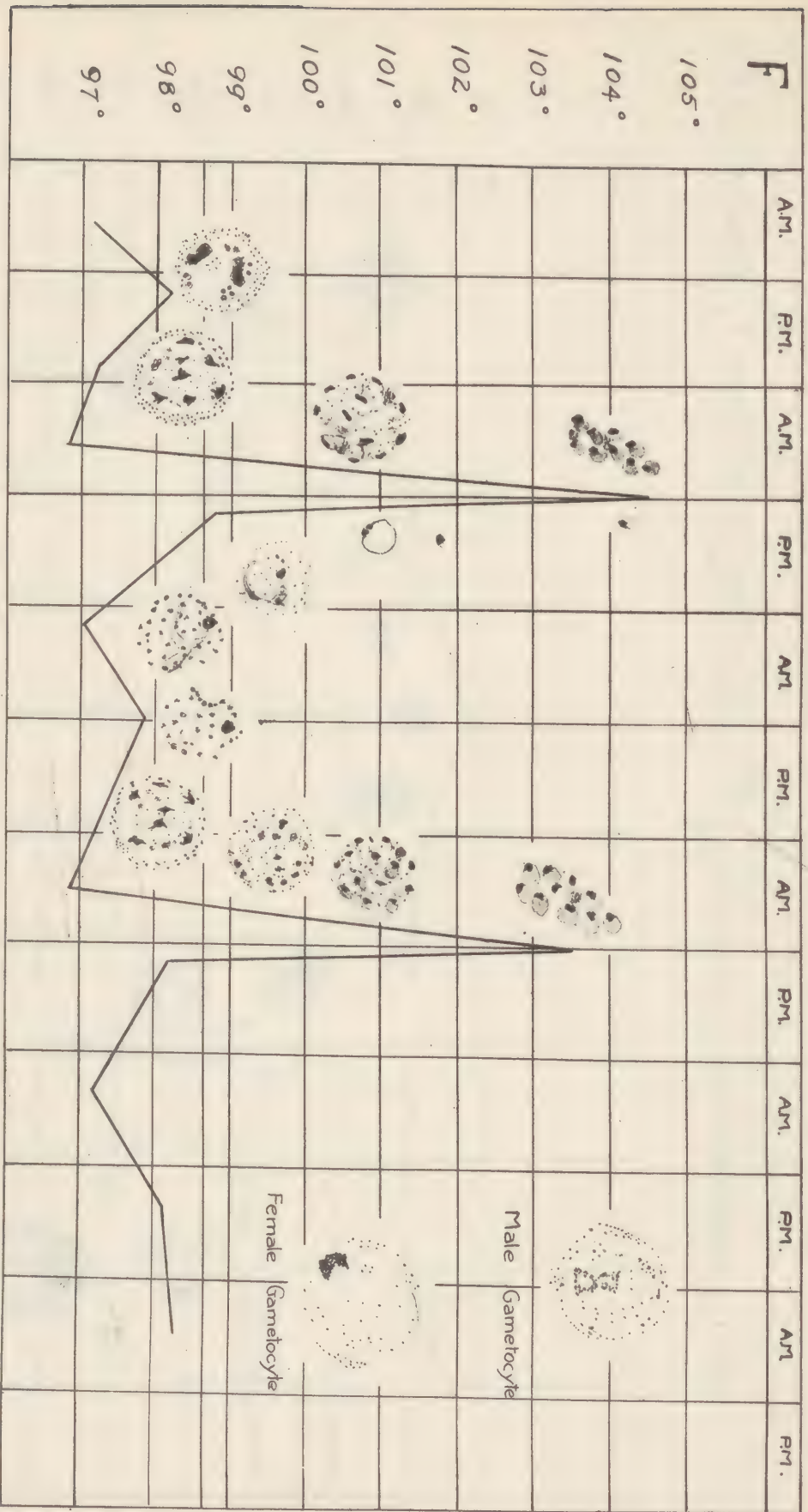
Early diagnosis and treatment is of utmost importance if a favorable prognosis is to be expected in P. falciparum infections, owing to its tendency to produce early and unexpected cerebral complications. Therefore, if P. falciparum infections have been found in the laboratory in which you are working and you have a blood smear in which you are certain there are malarial parasites, but you are unable to determine the type, report the case as positive for malaria so that treatment may be started. Then make additional smears and study carefully to determine the type.





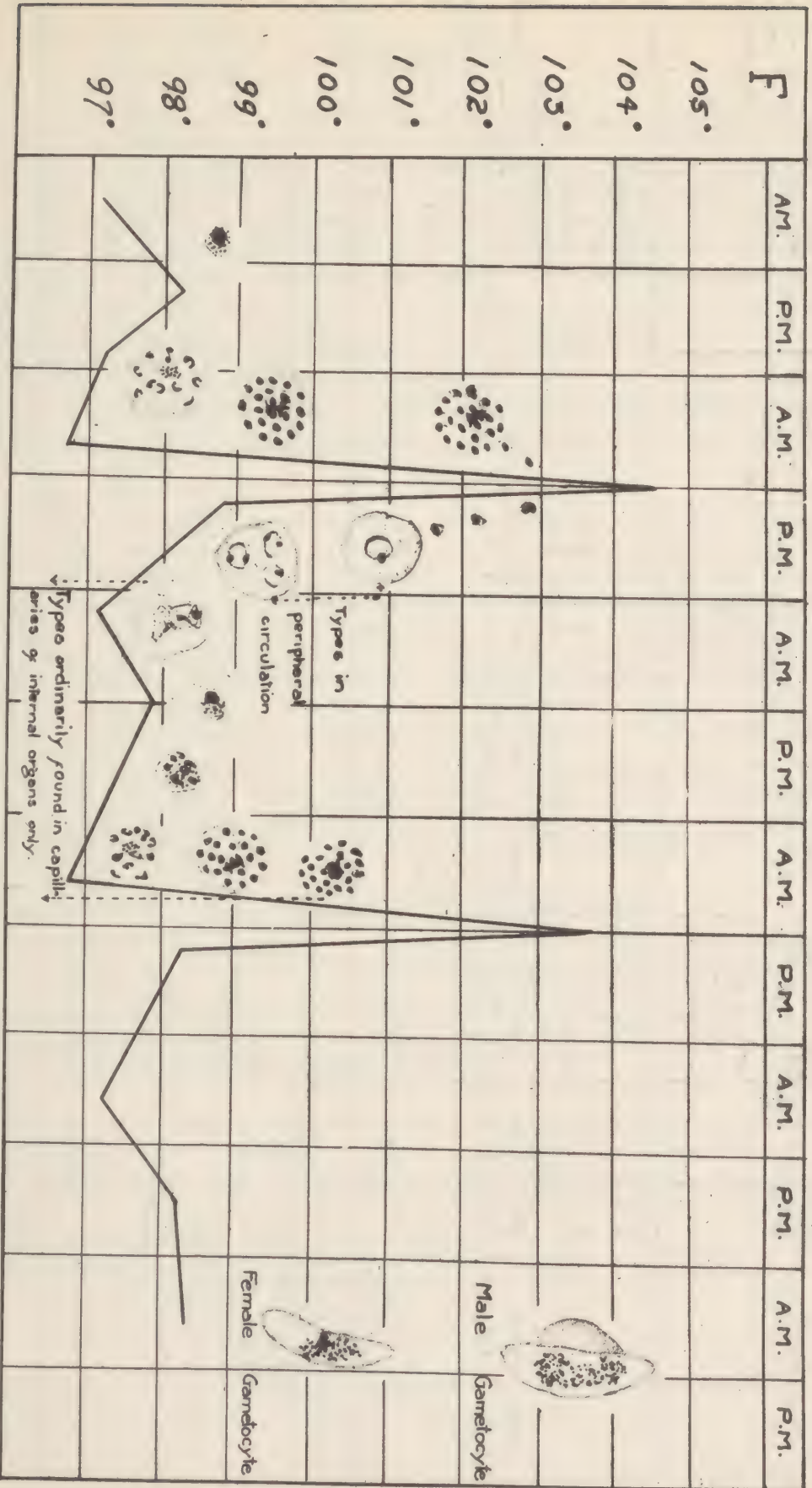


Life Cycle of *P. malaria* in Man Showing Relation to Temperature Curve.



Life Cycle of *P. vivax* in Man Showing Relation to Temperature Curve.





Life Cycle of *P. falciparum* in Man Showing Relation to Temperature Curve.

Parasitized Red Cells				Schizogony										Gametogony			Sporogony		Seasonal prevalence in sub-tropical regions where all 3 types of malarial parasites are present.		Total admissions (absolute numbers) U.S. Army 1920-1930 inclusive		Total deaths (absolute numbers) U.S. Army 1920-1930 inclusive	
Species	Size	Color	Shrink- ing	Resistance to Multiple Infection	Size		Motil- ity	Length of Life Cycle	Number of Nero- zoites formed	Stages present in peri- pheral blood at any given time	Pigment			Differen- tial char- acters of schizonts	Size		Shape	Length of Life Cycle	Time re- quired to complete cycle at 20°C Relative Humidity 70					
					Ring forms first seen Hour	Mature forms in per- centage R.C.C.					Res- cence	Type	Color		d	d								
<i>P. vivax</i> (tertian or benign tertian)	Un- stained	Pale yellow- ish red ules	Fine gran- ules	Un- common	15 μ to 17.5 μ	Fills cell	Active ameboid	48 hours	12 to 16	All stages, but one predom- inates	yes Fine grains	Light Brown	See illus- trations.	7 μ to 9 μ to 8 μ to 10 μ	d Round or d Oval	Four Days. Inject- ive for mos- quitos in 7 days	7 to 12 days	Spring and early Summer	10,714 (75.1%)	2 (8.32%)				
	Stained		Pale pink brown- ish red ules of Schryver																					
<i>P. malarie</i> (quartan)	Un- stained	Dull green present	Not	Un- common	2 μ to 2.5 μ	Fills cell	Slugg- ish ameboid	72 hours	8 to 12	All stages, but one predom- inates	Large ant. coarse or grains	Dark brown or black	Band Form. See illus- trations	5.5 μ to 6 μ to 5 μ to 7.5 μ	d Round or d Oval	Seven days. Inject- ive for mos- quitos in 10 days	20 to 24 days	Late Fall	128 (0.9%)	1 (4.16%)				
	Stained		green- ish red base- or pink normal granules																					
<i>P. falciparum</i> (tertian, malignant tertian, or pernicious tertian)	Un- stained	Drusy coarse Copper grains	Fair	Fairly common	0.5 μ to 1.5 μ	Fills only	Little or None	48 hours	12 to 32	Ring forms and gametocytes only. Other forms in capillaries of internal organs except in severe cases	Yes Coarse grains	Dark brown or black	See illus- trations	12 μ to 14 μ to 15 μ to 16 μ	Crescent shaped with the male end more rounded	Four days. Inject- ive for mos- quitos in 7 days	16 to 20 days	Late Summer and Early Fall	3,432 (24%)	21 (87.62%)				
	Stained		Pinkish red or shapling of malar normal stains dark purple																					



## MEDICAL ENTOMOLOGY

## INTRODUCTION

Medical entomology is the study of insects and insect-like animals (arthropods) and their relation to human disease and discomfort. They are of special interest to the medical soldier because of their association with a number of diseases of importance to troops in garrison, camp or campaign. The principal disease-relationship of these forms is that of transmission: malaria and yellow fever by certain mosquitoes, plague by fleas, and typhus by lice. Also of importance are the arthropods that cause disease directly, they themselves serving as pathogenic organisms. The itch-mite of man, for example, invades the human skin and produces a severe irritation; this disease (scabies: "the itch") caused thousands of men to be admitted to hospitals during the World War. Many arthropods, as nuisances, cause discomfort to troops, such as the annoyance provoked by the common house flies and mosquitoes, especially when they occur in large numbers.

Due to the widespread distribution of arthropods, and to their close association with troops at all stations, it is necessary that army personnel be prepared to determine whether the species present in a locality are likely to be of medical importance. This may entail collection of representative forms, making a tentative identification, and if certain specimens are likely to be important, forwarding them to large central laboratories where positive identification may be accomplished. The medical technician is not expected to know all of the various species by their scientific names, but if called upon to send in a representative sampling, for example, of mosquitoes in the vicinity, he should be able to send mosquitoes, and not a various assemblage of small beetles, flies, midges, fleas, moths, etc. It is the purpose of this section to familiarize the medical technician with the medical importance of various arthropods and to aid him in roughly identifying the forms.

## CLASSIFICATION OF ARTHROPODS

Classification of arthropods is based upon a system of organization. As an army is divided into corps, which are further divided into divisions, then brigades, regiments, battalions, companies, etc., down to the individual men, similarly animals are grouped into phyla, which are subdivided into classes, orders, families, genera and species.

For the purpose of the medical soldier the phylum Arthropoda may be divided into four classes, viz., Insecta (insects), Arachnida (ticks, mites, spiders, scorpions, etc.), Myriapoda (centipedes and millipedes) and Crustacea (crayfish, shrimp, etc.). As will be seen below, these classes can be further divided again and again until the individual species are reached. As an example, the yellow fever mosquito may be classified as follows:

Phylum - Arthropoda  
Class - Insecta  
Order - Diptera  
Family - Culicidae  
Genus - Aedes  
Species - Aegypti



The scientific name of an animal is a combination of the names of the genus and the species: the scientific name of the yellow fever mosquito is Aedes aegypti.

#### PROCEDURE IN IDENTIFICATION OF SPECIMENS

Since all members of any of the above classes possess certain common characteristics that differentiate them from the other classes of arthropods, use can be made of these characters in making "classification keys". These keys enable the worker to identify specimens. A working example of such a key follows:

1. If the specimen has 3 or 4 pairs of legs, go to ..... 2  
     If it has numerous pairs of legs, go to ..... 3
2. With 3 pairs of legs (Fig. 7) ..... Insecta, see p. 5  
     With 4 pairs of legs (Fig. 4) ..... Arachnida, see p. 3
3. Lives in water (Fig. 1) ..... Crustacea, see p. 2  
     Lives on land (Fig. 2) ..... Myriapoda, see p. 2

An examination of the above key will reveal that identification of specimens is not too difficult a matter. It is simply necessary to "run it through the key". If by running the specimen through the key it should prove to be an arachnid, it is only necessary to turn to the section on Arachnida where a key to this class will be found. By subjecting the specimen to several such keys, identification can be done.

The key is only an aid, not a final proof of identification. Since in the insect class alone there are thousands of species, it is obvious that the keys and outlines in this manual will not serve to identify all specimens. They will, however, enable the worker to identify many forms, even though the identification may not be entirely accurate in all cases. For more satisfactory identification, this manual should be supplemented by standard texts, and for absolute identification specimens may be transmitted to entomological centers (See page 13).

#### CRUSTACEA OF MEDICAL IMPORTANCE

Crustaceans (crayfish, shrimp, etc.) are of little importance to the medical soldier, but are worthy of mention because of a few species are associated with human disease. Several species of microscopic forms serve as intermediate hosts in the transmission of certain intestinal worms. A typical crustacean is illustrated in figure 1.

#### MYRIAPODA OF MEDICAL IMPORTANCE

Myriapods include the centipedes (Chilopoda) (Fig. 2) and the millipedes (Diplopoda) (Fig. ). They may be differentiated by the number of legs on each body segment, centipedes possessing one pair of legs per segment, whereas millipedes have two pairs on each segment.



## From Patton and Evans, "Insects, Ticks, Mites and Venomous Animals".





Millipedes may for practical purposes be omitted from the list of offenders. They have no fangs and are, therefore, harmless so far as venomous species are concerned.

Certain Centipedes, on the other hand, are very important. Although nearly all of the species possess fangs, they are for the most part unable to penetrate the human skin. While no deaths have been recorded from centipede-bite, painful injury can be accomplished. Species of Scolopendra, Geophilus and Lithobius are capable of injury.

#### ARACHNIDA OF MEDICAL IMPORTANCE

The class Arachnida is very important from a medical standpoint, containing many species that serve as transmitters of disease, as well as species that cause disease directly. The following key will serve to separate the more important orders:

1. With abdomen divided into segments..... 2  
    Abdomen not divided into segments..... 4
2. Body divided by constriction into two main parts (Fig. 4)..Spiders, p.3  
    Body not divided by such a constriction..... 3
3. Minute species (smaller than a pin-head in size) (Fig. 5)....Mites, p.4  
    Medium-sized species (larger than a pin-head in size) (Fig.6)Ticks, p.4
4. With spine at tip of tail (Fig. 3A).....Scorpions, p.3  
    Without spine at tip of tail (Fig. 3B).....Whip Scorpions, p.3

Scorpions (Fig. 3A) are offensive to man because of their sting, which is accomplished by a spine at the tip of the tail (abdomen). Although many of the smaller species are harmless because they are not able to penetrate the human skin, some species, particularly of the genus Centruroides are very important. In the city of Durango in northern Mexico one species, C. suffusus, causes on the average of 50 deaths per year. The 20 or more common species of the southern United States are generally capable of producing only a painful sting. Of interest to soldiers is the fact that some scorpions have a tendency to crawl into shoes during the night.

Whip Scorpions (Fig. 3B) are very ferocious in appearance, but are entirely unimportant from a medical standpoint. Their only interest to soldiers is the tendency to confuse them with scorpions.

Spiders.- Although all spiders (Fig. 4) produce venom, only a few possess fangs sufficiently powerful to penetrate the human skin. Of most importance to the soldier is the "black widow" spider, Latrodectus mactans. This is a small black spider, which can be distinguished by the reddish hour-glass marking on the underside of its abdomen. It may be found in grass, shrubs, old out-houses and privies. Its bite produces severe symptoms and in some cases death.

Tarantulas present a ferocious appearance, but compared to the "black widow" their bite is mild. Their hairy bodies serve as a convenient resting place for many bacteria, and secondary infection of the wound is common.



**Mites.**- All species of mites (Fig. 5) are very small, many being barely visible to the naked eye. In general only three forms are of medical importance. The Common itch mite of man, Sarcoptes scabiei, tends to be found where many people may be forced to live together under unhygienic conditions. In times of national emergency where many soldiers come in close contact with each other, and especially where bathing facilities are meager, cases of scabies may appear. When the mites attack, they usually invade the skin between the fingers, and spread to other parts of the body from these foci. The females burrow into the skin and lay their eggs in the tunnels made during their migrations. The intense itching which this skin invasion produces results in scratching, with subsequent secondary infection.

The genus Trombicula contains two very important species. The most important is the Japanese Chigger, T. akamushi, which transmits Japanese River Fever. This disease is very similar to Rocky Mountain Spotted Fever, and is as fatal to man. These mites generally occur on small rodents, especially field mice, which serve as reservoir hosts of this disease. These mites are widely distributed throughout certain parts of Japan.

The other species of medical interest is the American "chigger" or "redbug". These tiny mites, hardly larger than a pin point, are larval forms of Trombicula irritans. Since these larvae are very numerous in the fields during the late spring and early summer, troops working in high grass and weeds during warm weather are subjected to their attacks. The larvae, which have only three pairs of legs instead of four pairs, attach themselves to the exposed parts of the body where their bites produce an intense itching that may last several days. This species is widely distributed in the United States, and is a common cause of annoyance to soldiers.

**Ticks.**- The Ticks are important chiefly as transmitters of harmful organisms and incidentally as direct cause of disease. They have a widespread occurrence, particularly in the tropics and subtropics. For purposes of general classification, ticks can be divided into two groups: the soft-bodied ticks (family Argasidae) and the hard-bodied ticks (Ixodidae).

"Head" concealed beneath front margin of body, shield on upper surface of body absent (Fig. 6,A) ..... Soft-bodied ticks, p. 4

"Head" not concealed beneath front margin of body, shield on upper surface of body present (Fig. 6,B) ..... Hard-bodied ticks, p. 5

**Soft-bodied Ticks.**- The members of this group (Argasidae) (Fig. 6,A) are not fixed parasites as are the hard-bodied ticks (Ixodidae). Their habits are similar to bedbugs, visiting the host for a blood-meal, then returning to the cracks and crevices in which they live. Of the two genera that belong to this family, the genus Ornithodoros contains nearly all the species of medical importance. The members of this genus are very similar in appearance to those of the genus Argas, but may be differentiated since the edges of the body are rounded, whereas these edges are sharp-angled in Argas species. Eyes are frequently present in Ornithodoros and absent in Argas.



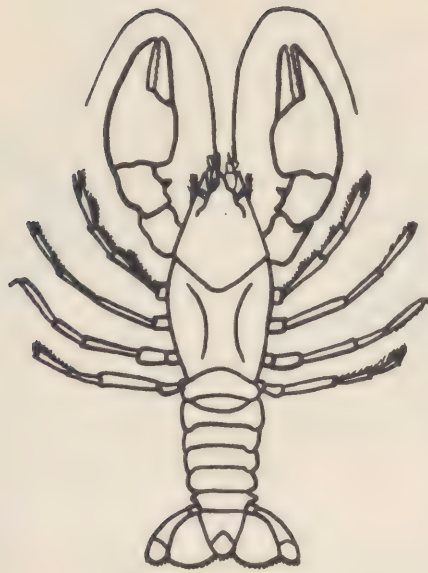
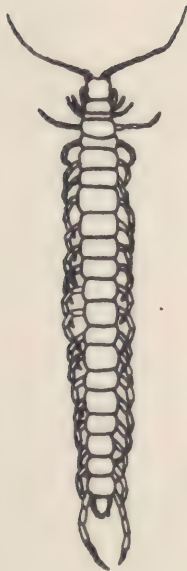


Fig.1. Crayfish (a crustacean)



**A**



**B**

Fig.2. Myriapods. A, Centipede; B, Millipede.





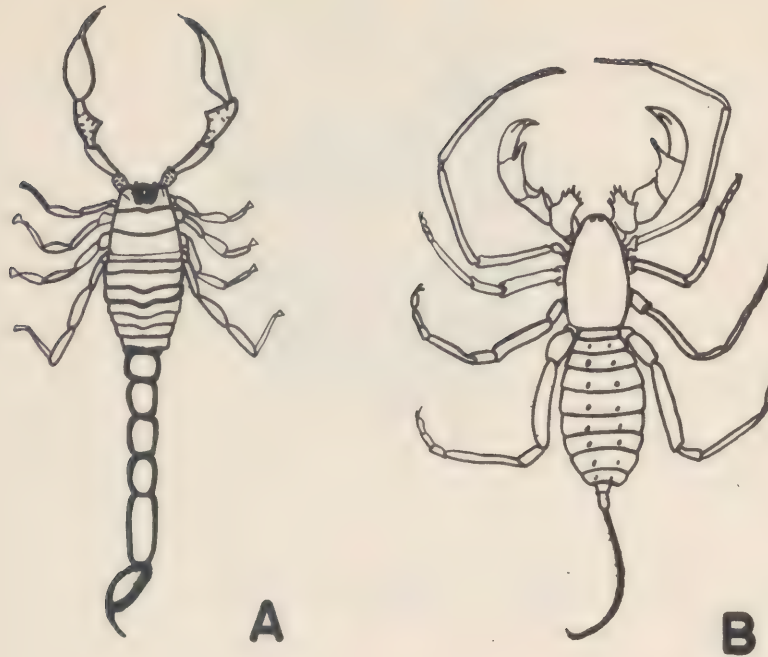


Fig.3. Comparison of scorpion and whip-scorpion.  
A, Scorpion; B, Whip-Scorpion.



Fig.4. Black widow spider (an arachnid).





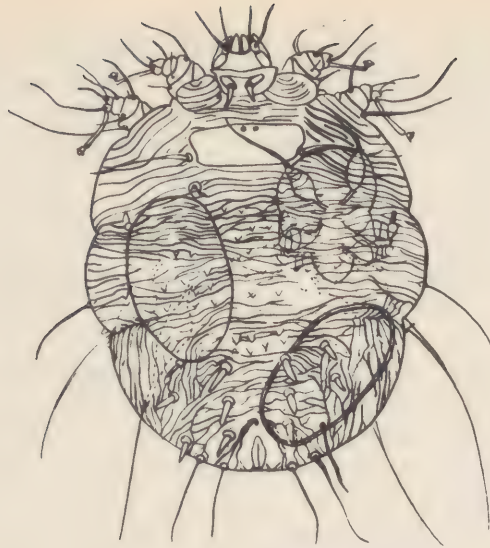


Fig.5. Itch-mite of man.

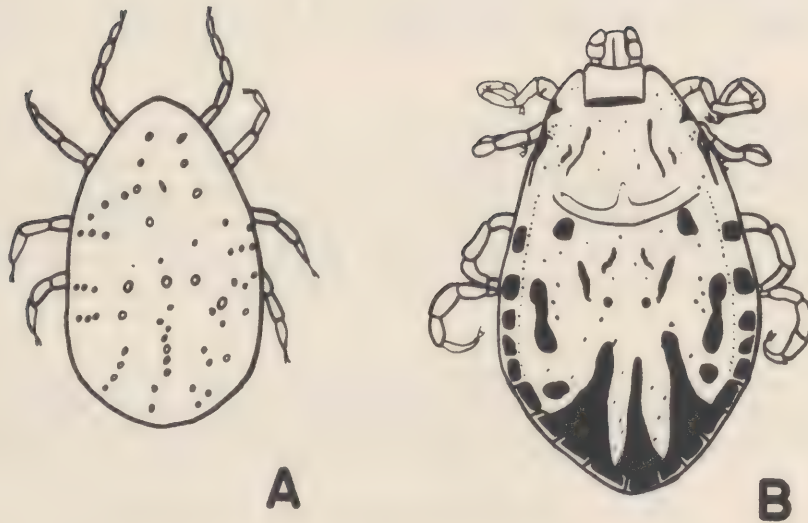


Fig.6. Ticks. A, Soft-bodied tick;  
B, Hard-bodied tick.





Soft-bodied ticks may transmit relapsing fever from animals, human cases or carriers to other animals or to man. The tick acquires the spirochaetes (Borrelia duttoni) when it takes a blood meal, harbors them for prolonged periods, and later infects the person (or animal) when it takes another blood-meal. Hereditary transmission from adult tick to its offspring, through the egg stage, may occur. Opossums and armadillos may serve as reservoirs from which the ticks acquire their infections. The more important species are Ornithodoros moubata in Africa, O. talaje in South and Central America and Mexico, and O. turicata in the Southern United States.

Hard-bodied ticks.- The members of this family (Fig. 6,B) differ in feeding habits from soft-bodied ticks in attaching themselves to their hosts and feeding for long periods of time. They are by far the more widely distributed group, being very well represented in tropical, subtropical and temperate regions. Of the eight genera belonging to this family, the genus Dermacentor is by far the most important. Others of importance are Haemaphysalis and Ixodes. In general, differentiation of the hard-bodied ticks is rather difficult, and for such identification the worker is referred to standard texts.

Hard-bodied ticks are found associated with various human diseases, the most important of which are Rocky Mountain spotted fever, tularaemia, and tick paralysis. Rocky Mountain spotted fever, an acute infectious disease of man having a high mortality, is caused by a rickettsial organism (Dermacentroxenus rickettsi). The tick of most importance in the transmission of this microorganism to man is the Rocky Mountain wood tick, Dermacentor andersoni. The North American rabbit tick, Haemaphysalis leporis-palustris, is important in transmitting the disease from rodent to rodent.

Tularaemia (rabbit fever), caused by Pasturella tularensis, may be transmitted to man by the bites of the wood tick D. andersoni. Again the rabbit tick, H. leporis-palustris, is important in transmitting the disease among rodents (rabbits).

In addition to transmitting disease, many ticks are able to inflict injury by the bites alone. Certain species particularly the wood tick, D. andersoni, and various species of Ixodes are offensive in this respect. The bites of these species cause a form of paralysis, and in a few cases, deaths have resulted. The exact mechanism by which the paralysis is effected is not known, but it is probable that the tick secretes some sort of neurotoxin into the wound.

At times the technician may be presented with small forms that resemble ticks in appearance, but have only three pairs of legs. These forms may be the larval stages (called seed ticks), and should not be confused with insects.

#### INSECTS OF MEDICAL IMPORTANCE

The class Insecta also contains many species of medical importance. It is in this group that are included such offensive forms as mosquitoes, lice, fleas, bedbugs, etc. The following outline will serve as a guide in classifying the



more important orders. Those orders of medical importance are marked with an asterisk (\*). For more complete information and keys the reader is referred to standard entomological texts.

Insect Order	Common names of Forms	Illustrations
* 1. Anoplura	lice	Fig. 8
2. Coleoptera	beetles, weevils	Fig. 7,B
* 3. Diptera	flies, mosquitoes, midges, etc.-	Figs. 9,10,11
* 4. Heteroptera	bedbugs, kissing-bugs, cicadas, etc.	Figs. 16, 17
5. Hymenoptera	ants, bees, wasps, etc.	Fig. 7,D
6. Lepidoptera	butterflies, moths, skippers	Fig. 7,C
7. Orthoptera	roaches, crickets, grasshoppers-	Fig. 7,A
* 8. Siphonaptera	fleas	Fig. 18

Only the above insect groups indicated as of medical importance will be discussed below. The other groups have been included in order that the technician may observe examples of those groups, and thus avoid confusion with important species. He should bear in mind, however, that this list is far from complete.

Anoplura (lice). There are two species of lice that infest man, viz. the head-louse and body-louse (Pediculus humanus) and the crab-louse (Phthirus pubis). The head-louse and body-louse are two varieties of the same species, and are called P. humanus var. Capitis and P. humanus var. corporis respectively. The head-and body-lice and the crab-lice are easily differentiated by the length of the body in proportion to its width. Both varieties of P. humanus are about 3 times as long as they are broad, whereas crab-lice are as broad as they are long. (See Fig. 8).

The head-louse lives among the hairs of the head of its host and attaches its eggs near the base of the hairs by means of a glue formed in a special gland. The hairs around the ears and back of the head are most frequently used as sites for depositing the eggs.

The body-louse usually infests the clothing along the seams, where it attaches its eggs to the fibers of the cloth. Woolen clothing seems to be the material of choice of the body louse, because the eggs may be easily attached to the wool fibers.

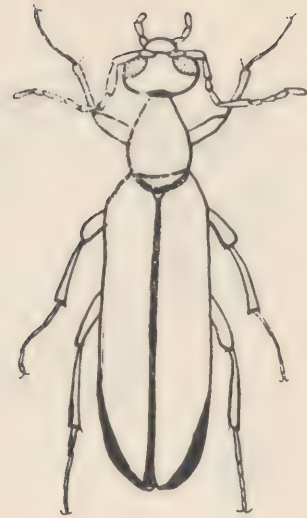
Man is affected by the head-and body-louse in two ways: by the direct, mechanical effect of the bites, and by their transmission of pathogenic organisms. The bites produce minute hemorrhagic spots which are accompanied by irritation, often with intense itching, leading to scratching and secondary infection.

Among the infectious diseases transmitted by lice are typhus, trench fever, relapsing-fever and plague. European or epidemic typhus which resulted in many deaths among soldiers in the European armies in the World War, is caused by a rickettsial organism, Rickettsia prowazeki var. prowazeki, transmitted by the head-and body-lice (especially the latter). Transmission may be accomplished





**A**



**B**



**C**



**D**

Fig.7. Insects. A, Cockroach(Orthoptera);  
B, Beetle (Coleoptera); C, Moth  
(Lepidoptera); D, Ant (Hymenoptera).





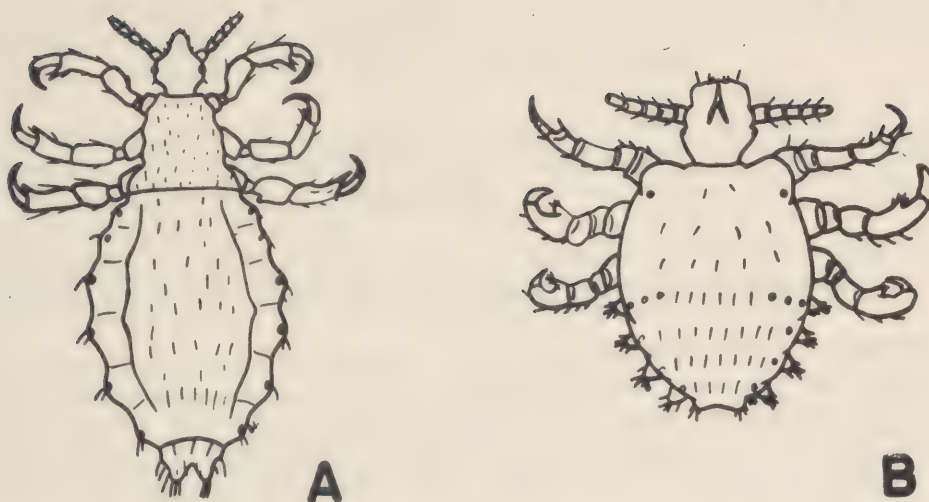


Fig.8. Lice(Anoplura). A, Body louse;  
B, Crab louse.



Fig.9. Flies. A, Larva of house-fly;  
B, Pupa of house-fly.





in three ways--by deposition of the louse's feces on the injured skin; by crushing the insect against the skin and by its bite. Trench fever is caused by Rickettsia quintana and transmitted by the bite of the body louse.

A relatively mild type of relapsing fever caused by the spirochaete, Borrelia recurrentis, is transmitted by lice when infective lice are crushed on the skin. The disease is not transmitted by the bite.

It has been shown recently that lice on marmots in western Montana were naturally infected with the organism of plague, Pasturella pestis.

The crab-lice generally frequents the pubic hairs, but it has been found also on other hairy parts of the body, the legs, arm-pits, beard, eye-brows and eye-lashes. As in the case of the head-lice, the crab-lice lays its eggs attached to the hairs of the host. This insect provokes some local irritation, but has not yet been incriminated in the transmission of any disease.

Diptera (flies, mosquitoes, etc.).- The Diptera is another arthropod order which contains species that may serve both to transmit disease and to cause it directly. The importance of some mosquitoes and tsetse-flies in malaria, and sleeping sickness is common knowledge, but there are many other mosquitoes and "biting" flies that carry disease. There are many species living and breeding in close contact with army personnel that may cause disease directly. It is well known that in their life-cycles flies pass through egg, larval, pupal and adult stages (Fig. 9). The larvae ("maggots") of some species may gain entrance to the human body and invade the tissue, such an invasion of tissue with fly larvae being spoken of as "myiasis".

Since the order Diptera contains so many species with variance of form and habit, it is impossible to present a simple key to all the major groups. The following key-outline will, however, serve as a convenient guide in separating the forms. It should be remembered that this outline is one of convenience, and for a more accurate classification standard texts should be consulted. Since the mosquitoes constitute the most important dipterous groups, their classification is treated in more detail, and the technician should be able to make tentative examination of kinds of mosquitoes even though the other forms may be regarded more lightly. Separate any specimens collected as follows:

1. Fragile (mosquito-like) (Fig. 13,A)..... 2  
    Stout-bodied (house-fly and horse-fly-like) (Fig. 13,B)... 3
2. Scales on wings (Fig. 11).....Mosquitoes, p. 7  
    No scales on wings (Fig. 13).....Mosquito-like Insects, p. 8
3. Biting flies (Fig. 14,A).....Stout-bodied Biting Flies, p. 9  
    Non-biting (Fig. 14,B)...Filth and Myiasis-producing Flies, p. 10

Mosquitoes. The family Culicidae to which the mosquitoes belong may be divided into three subfamilies. Of these latter groups the mosquitoes are the only ones that suck blood and are of medical interest. They may be easily distinguished from other common mosquito-like insects by the presence of scales on the veins and margins of the wings. (Fig. 11)



Mosquitoes are classified into several genera, most of which will be omitted since they are unimportant as disease-transmitters. The forms mentioned in this manual are the malaria-mosquitoes (genus Anopheles), the yellow-fever mosquitoes (genus Aedes), and the common house-mosquitoes (genus Culex, etc.).

The adult anophelines (genus Anopheles) can generally be differentiated from the adult culicines (genera Aedes, Culex, etc.) by the presence of spots on the wings (Fig. 10), these spots being absent in the latter groups. Other characters that serve to differentiate anophelines and culicines are the position of the body when at rest (Fig. 10) and the structure of the mouthparts, especially the palpi (Fig. 10). Differences between eggs, larvae and pupae of anophelines and culicines are illustrated in figure 10.

To a certain extent members of the genus Aedes can be differentiated from the other culicines by the presence of white bands on the legs, this character being generally absent in the others. The character is, however, not a valid one, and is given here merely as a suggestion in tentative classification. For more accurate identification the worker is referred to standard texts.

Malaria, a common, sometimes fatal, infection caused by various protozoan parasites (Plasmodium) and discussed elsewhere in this manual, is transmitted by mosquitoes of the genus Anopheles. Among the more important species are A. quadrimaculatus and A. maculipennis in the United States, A. albimanus and A. pseudopunctipennis in Mexico, Central America, the West Indies and northern South America, and A. minimus in the Philippines. The wings of several common species are illustrated in figure 11. Larval characters are shown in figure 12.

Yellow fever is a very fatal virus disease which at times may reach epidemic proportions. It is transmitted by Aedes aegypti. Although many other species of Aedes have been experimentally incriminated in the transmission of yellow fever, A. aegypti is the only one proven to transmit the disease naturally.

Dengue ("Breakbone fever") is a virus disease transmitted by mosquitoes. Although not a very fatal disease, during epidemics it causes a great deal of disability. Among the more important transmitters of this disease are Aedes aegypti and A. albopictus.

Filarial worm infections are also transmitted by mosquitoes. In these diseases the mosquitoes serve as intermediate hosts, and when feeding on a person the filarial larvae are transferred to the human host. Although the common house-mosquito of the Southern states (Culex quinquefasciatus) is usually said to be the principal vector in filarial infections, many other species included in several genera have been incriminated.

Mosquito-like Insects.- These are very small hairy flies that may be confused with mosquitoes. They are differentiated from that group, however, by the absence of scales on the wings.



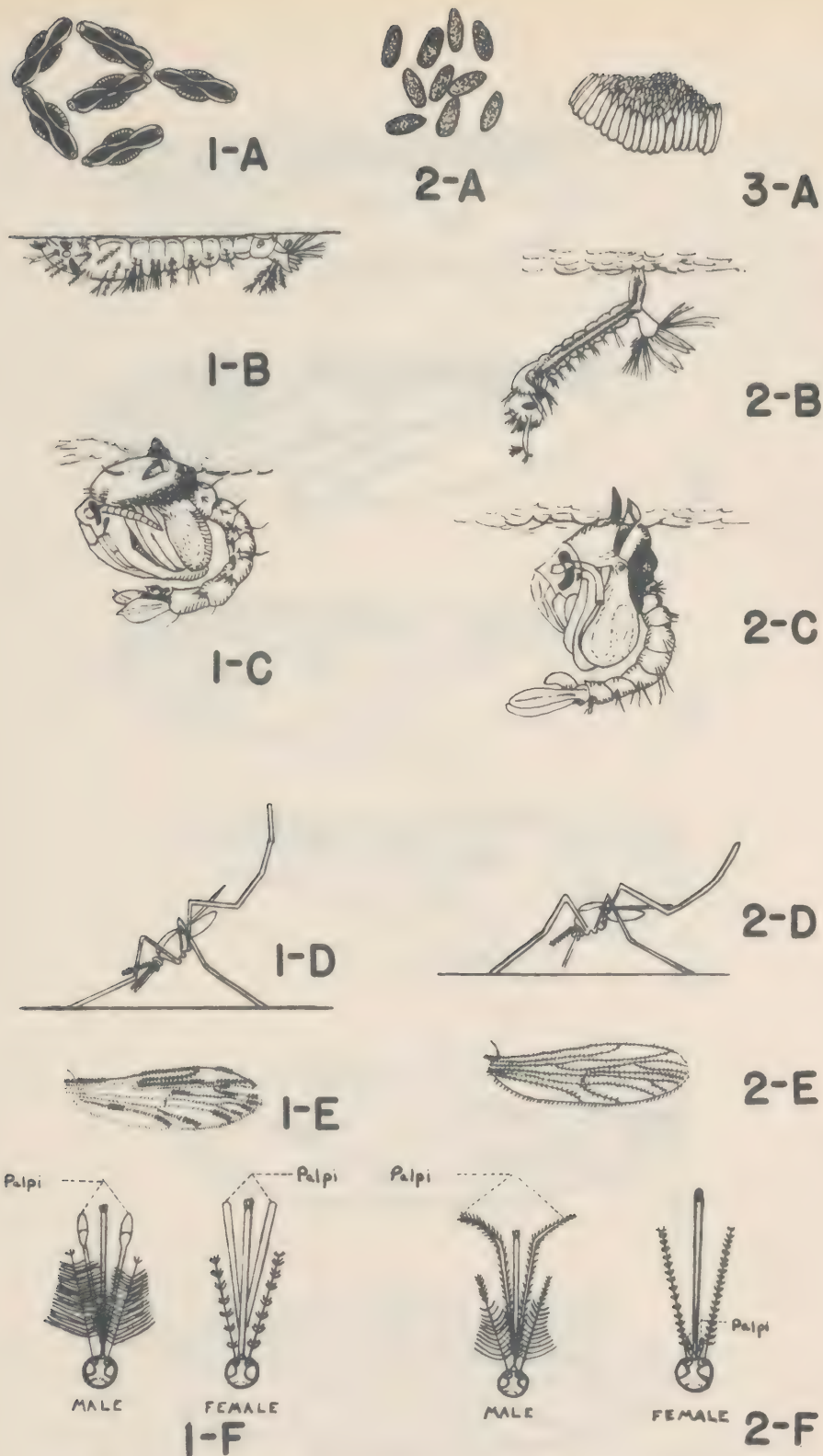


Fig. 10. Mosquitoes. Comparison of various stages of anopheline and culicine mosquitoes. 1. *Anopheles*, A, eggs; B, larva; C, pupa; D, adult; E, wing of adult; F, mouthparts of adult male and female. 2. *Aedes*, A, eggs; B, larva; C, pupa; D, adult; E, wing of adult; F, mouthparts of adult male and female. 3. *Culex*, A, typical raft of eggs.





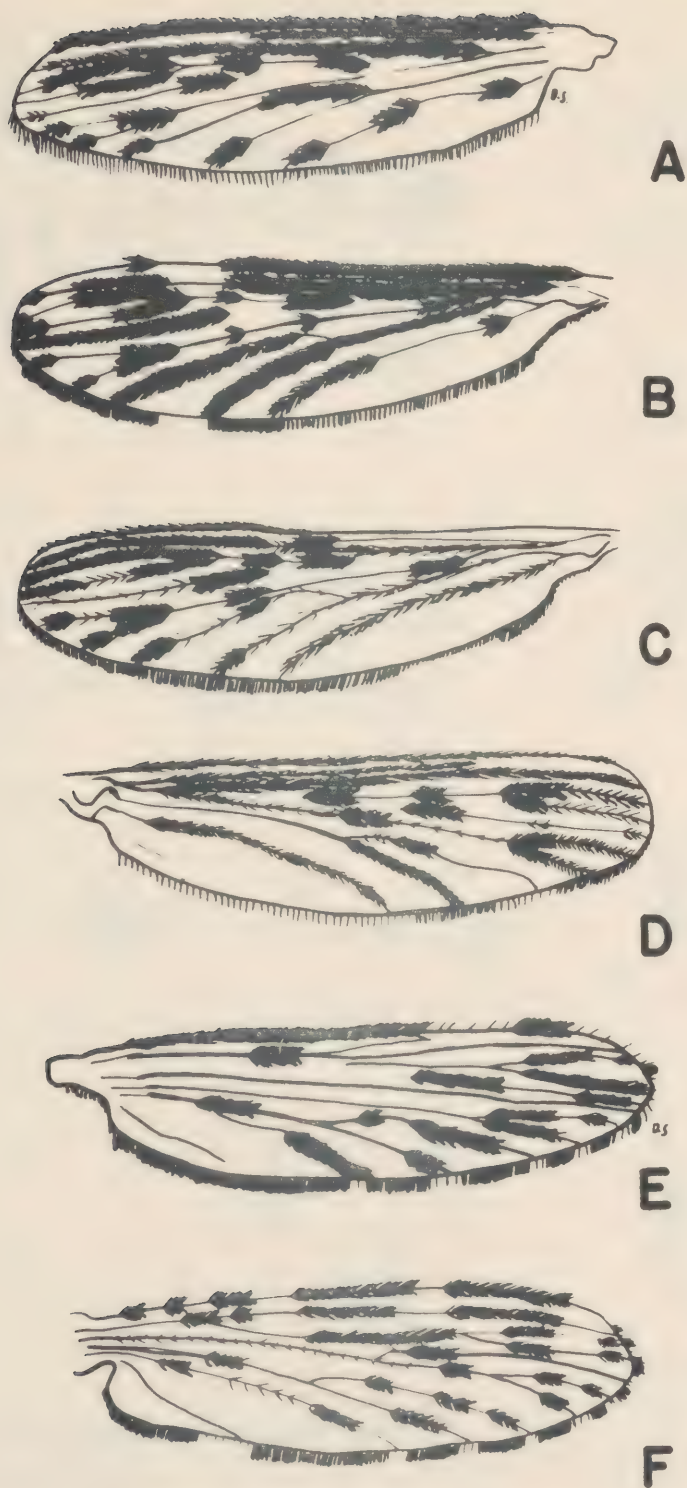
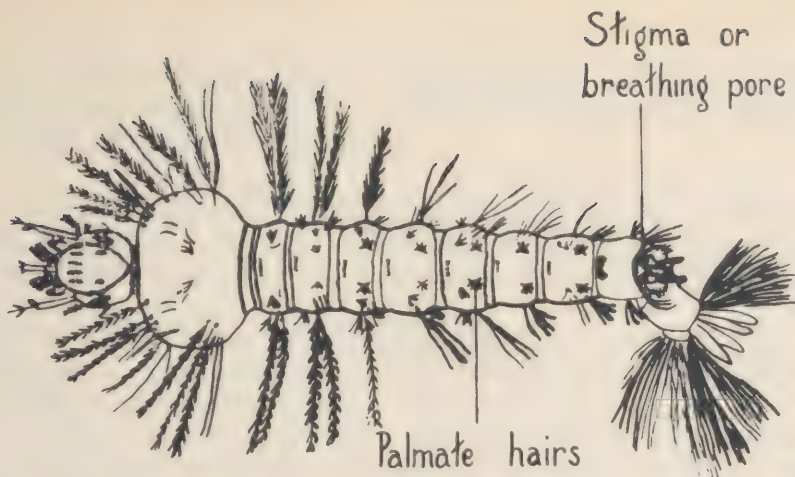


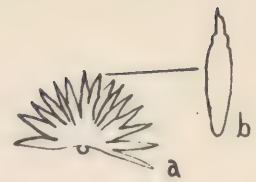
Fig. 11. Wings of *Anopheles* mosquitoes. A, *A. crucians*; B, *A. punctipennis*; C, *A. maculipennis*; D, *A. quodrimaculatus*; E, *A. pseudopunctipennis*; F, *A. albimanus*.



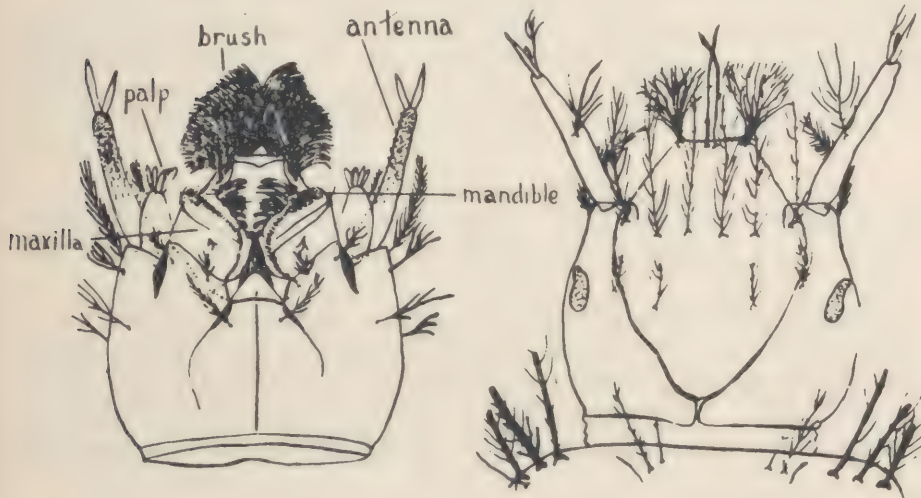




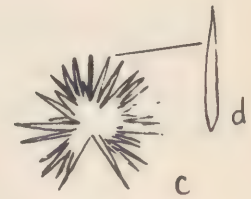
Dorsal view of an Anopheline Larva



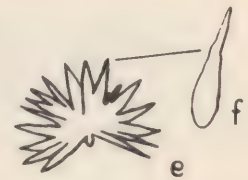
*A. quadrimaculatus*



Ventral and Dorsal views of an Anopheline Larva



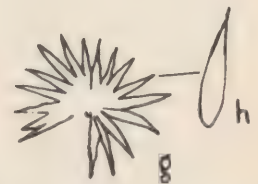
*A. albimanus*



*A. punctipennis*



Hypopygium of male *Anopheles albimanus* and  
*A. punctipennis*



*A. pseudopunctipennis*

Fig.12. Anopheles mosquitoes. Structural characteristics of larva. Hypopygia of males.



The sand flies (family Psychodidae) include several species of the genus Phlebotomus, some of which transmit "Pappataci" fever, kala azar, tropical sore, and some other diseases.

The gnats (family Simuliidae) include one group of medical interest, the genus Simulium. Certain species are instrumental in the transmission of onchocerciasis in Africa, Guatemala and southern Mexico.

The midges (family Chironomidae) are most liable to be confused with mosquitoes. The larvae of this family are the familiar "blood worms" found so often in stagnant water. The medically important genus is Culicoides, since many of the species are very annoying biters. Of even more importance is the fact that species of Culicoides transmit a filarial worm from man to man throughout tropical Africa.

The crane-flies (family Tipulidae) are of no medical importance but are mentioned in passing, (Fig. 13,A). These large mosquito-like forms are commonly encountered in warm weather and are almost always thought by the layman to be "large mosquitoes". They may be differentiated from mosquitoes quite easily, since they have no scales on the wings.

Stout-bodied Biting Flies.- This group includes the horse-flies, deer-flies, stable-flies, and tsetse-flies. The species of this group are of interest due to disease transmission as well as annoyance from their bites.

The horse-flies (Tabanus) and deer-flies (Chrysops) (family Tabanidae) (Fig. 13,B) are voracious blood-feeders. In addition to this annoying habit, which is limited to the females, species of Chrysops serve as transmitters of a filarial worm infection and of tularemia. Species of Tabanus have been recorded as transmitting anthrax to man and tularemia to guinea-pigs.

The stable-fly, Stomoxys calcitrans (family Muscidae), is a biting-fly that resembles the common house-fly very much in appearance. Occasionally before a rain it will be noticed that the "house-flies" are very annoying, and may even bite. When this occurs, it is not the house-fly as is commonly believed, but the stable-fly. They may be differentiated from house-flies by their biting mouthparts (Fig. 14). It has been suggested, but not proven, that the stable-fly is a vector of poliomyelitis, anthrax and tetanus. Both males and females feed on human beings.

The Tsetse-flies (family Muscidae) are of extreme importance in Africa; certain species of the genus Glossina being known to transmit Gambian and Rhodesian sleeping-sickness. Both males and females take blood-meals. The trypanosomes causing sleeping-sickness may be transmitted directly through mere mechanical action, or after undergoing a developmental cycle in the tsetse-fly. These flies are unique in depositing larvae rather than laying eggs.



Filth Flies and Myiasis-producing Flies.- This group includes the house-flies, flesh-flies, blue and green metallic-colored flies and other species usually associated with decaying filth and garbage. These flies are of interest because they produce disease in two ways, by mechanical transmission of harmful organisms or by direct invasion of the human body. The mechanical transmission of disease is easily accomplished by the flies' habits of frequenting filth, garbage, excreta, etc. The house-fly is important in the direct transmission of many human diseases, particularly typhoid fever, and including dysentery, tuberculosis, cholera and anthrax. In addition to transmitting disease by passing from infectious material to food, it lays eggs in the infective filth, and the maggots and adults that follow are infected with the pathogenic organisms.

The house-fly (Musca domestica) is the most important member of this group. Others are the flesh-fly (Sarcophaga), the non-biting stable-fly (Muscina stabulans), the lesser house-fly (Fannia canicularis) and the blue and green metallic-colored flies (Cochliomyia, Lucilia and Calliphora).

In addition to transmitting human disease, some of the above, along with other species, serve to infect man directly. This is accomplished by the gravid females depositing eggs or larvae on the skin or mucous membranes. The eggs hatch and the larvae penetrate the tissues, causing myiasis.

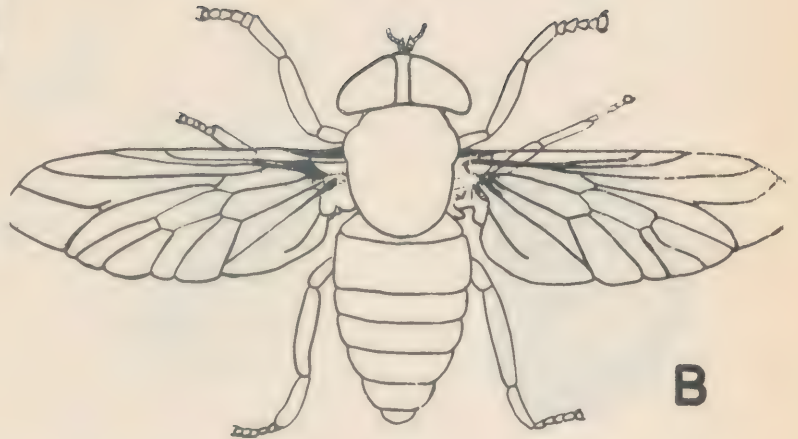
In many species laying of eggs on the human host is purely accidental, or the females may have been attracted to a sore or open wound, but in other forms deliberate attempts are made to deposit eggs on certain parts of the body that the larvae may hatch and invade the tissue. Another not uncommon method of infection is for a person to eat food upon which a fly has deposited eggs. The larvae hatch, and if present in considerable numbers, may produce severe irritation of the intestinal mucosa. These larvae may be found in feces sent to the laboratory for examination and should not be confused with parasitic worms. Some species lay eggs in the nostrils, and the "maggots" developing in the nasal passages have been known to cause extensive tissue damage and even death. Although in many cases the sites of invasion of these maggots serve as sites for the entrance of harmful bacteria, some fly larvae are known to keep the wounds very clean. Maggots living in the wounds of soldiers injured in battle have been noted to remove the debris and bone fragments and thereby promote rapid healing, the basis of the "maggot treatment" of certain types of wounds.

Among the more important myiasis-producing flies are the flesh flies (Sarcophaga and Wohlfahrtia), screw-worm fly (Cochliomyia), blow-fly (Calliphora), green-bottle fly (Lucilia), house-fly (Musca), warble-fly (Dermatobia), bot fly (Gastrophilus), lesser house-fly (Fannia), cattle bot (Hypoderma) and sheep bot (Oestrus).

Identification of the adult flies is rather difficult and will not be attempted herein, but reference made to standard texts. Maggots (Fig. ) are likely to be recovered in stool examinations, and may be presented for identification from breeding areas around army camps. These larvae, especially in the stage before pupation, can be identified by the pattern of the stigmal plates.

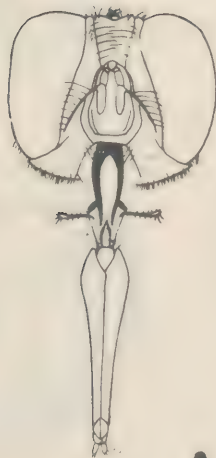


**A**

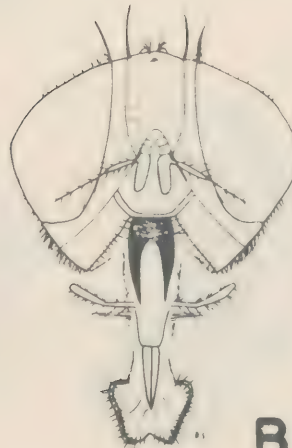


**B**

Fig. 13. Flies. A, Crane-fly; B, Horse-fly.



**A**



**B**

Fig. 14 Fly mouthparts. A, Head of stable-fly showing biting mouthparts; B, Head of house-fly showing non-biting mouthparts.





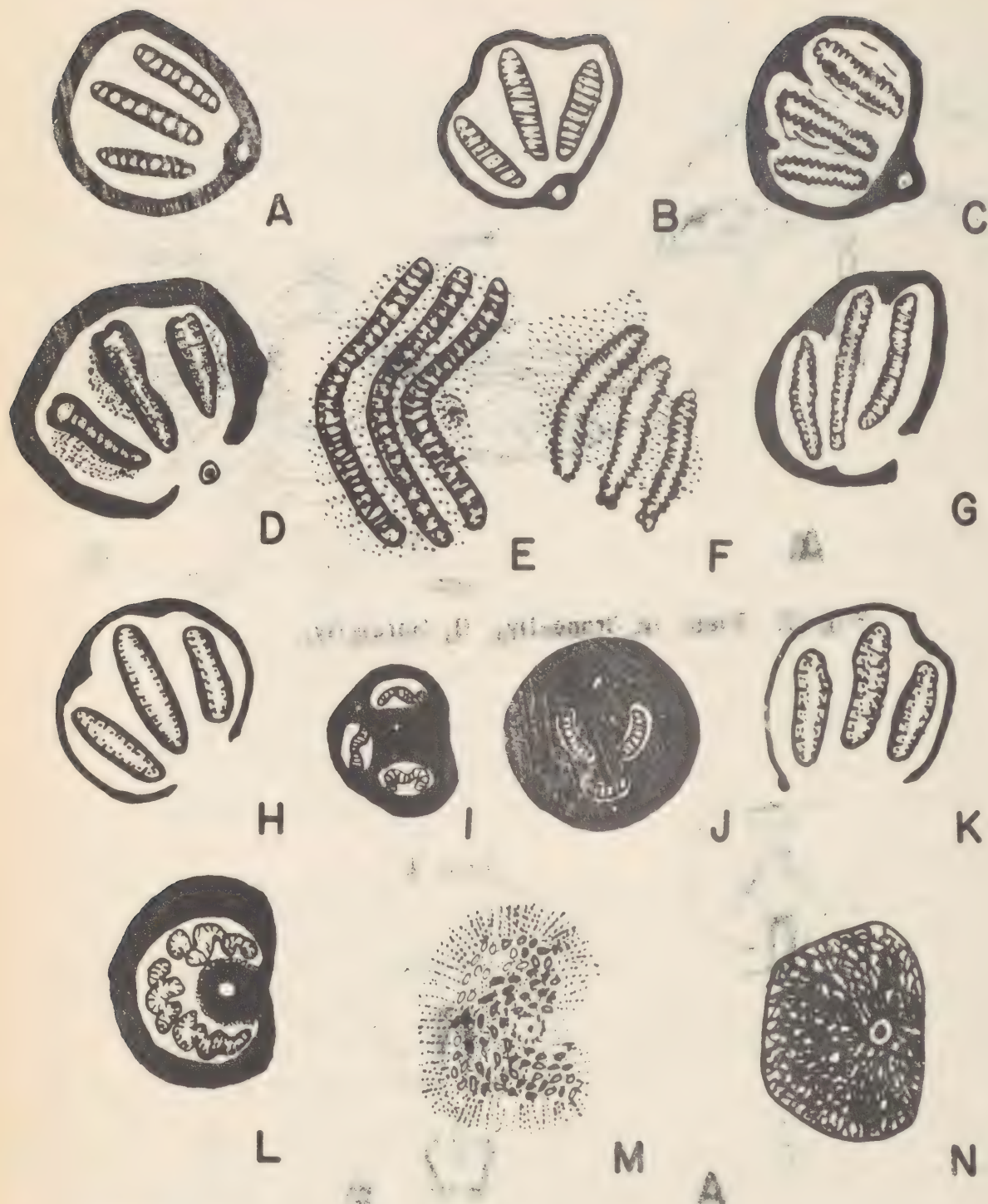


Fig. 15. Stigmal plates of fly larva. A, Blow-fly (*Calliphora*); B, Green-bottle fly (*Lucilia*); C, Blue-bottle fly (*Cynomyia*); D, Screw-worm fly (*Cochliomyia*); E, Bot fly (*Gasterophilus*); F, Warble fly (*Dermatobia*); G, Flesh fly (*Sarcophaga*); H, Black blow fly (*Phormia*); I, Biting stable fly (*Stomoxys*); J, Non-biting Stable fly (*Muscina*); K, Flesh fly (*Wohlfahrtia*); L, House fly (*Musca*); M, Cattle bot fly (*Hypoderma*); N, Sheep bot fly (*Oestrus*).

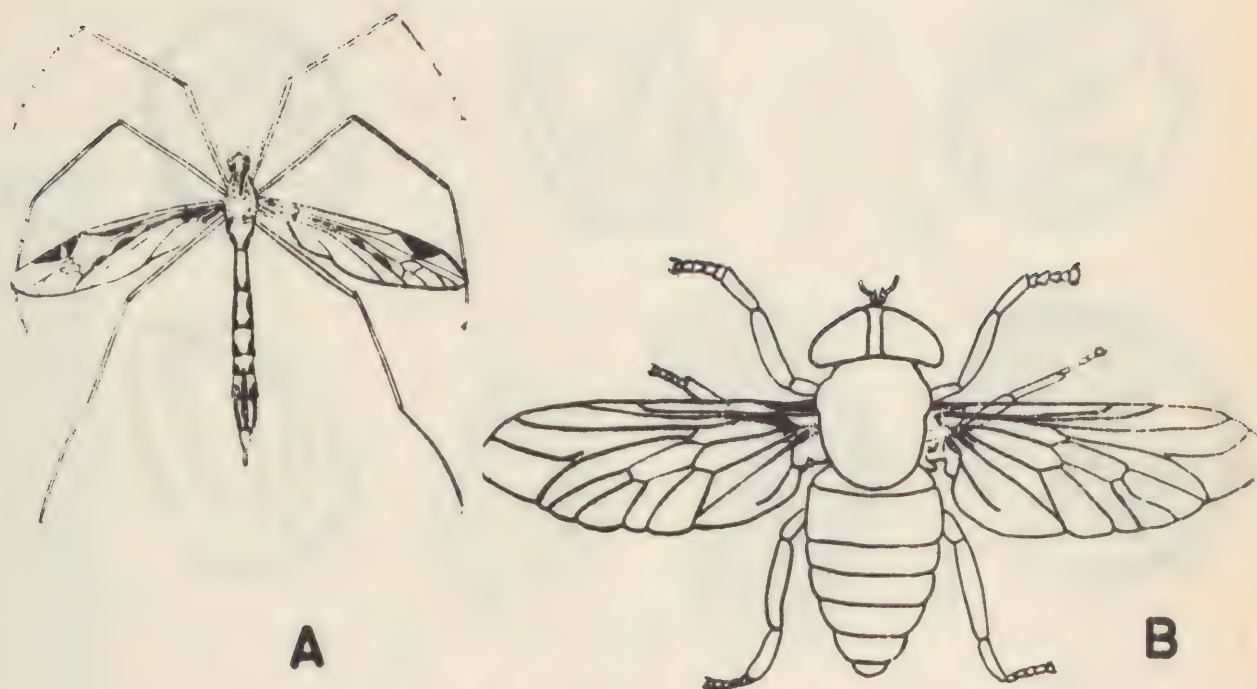


Fig. 13. Flies. A, Crane-fly; B, Horse-fly.

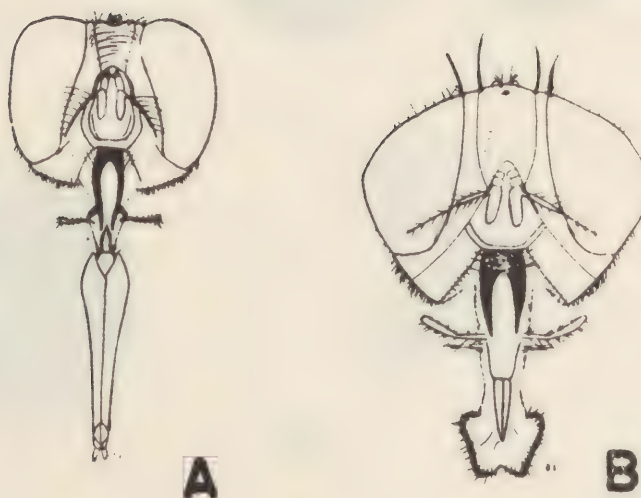


Fig. 14 Fly mouthparts. A, Head of stable-fly showing biting mouthparts; B, Head of house-fly showing non-biting mouthparts.



These plates are a pair of tiny chitinous structures located at the hind end of the maggot. They may be removed from the maggot and by using the microscope compared with those of the species illustrated in figure 15. In this figure only the left stigmal plate is illustrated. For descriptive details consult standard texts.

Heteroptera (bedbugs, kissing-bugs, cicadas, etc.). Although the term "bug" is applied to all members of the Insecta, strictly speaking only the members of the order Heteroptera are true bugs. The true bugs are very common, many living in water, while some abound on plants and feed on the juices. Some are blood-suckers and are very troublesome or even dangerous to man.

The common bedbug (Cimex lectularius) (Fig. 16) is world-wide in distribution and is a temporary parasite of man, feeding on his blood and living and breeding in the cracks and crevices of beds and other furniture, and in the walls and floors of his home. In the absence of the human host, bedbugs will feed on lower animals. Although the bedbug has been charged with the transmission of human disease, notably European relapsing fever, kala-azar, tularaemia and plague, there has been no definite proof, and its only interest to the medical soldier is the local irritation that some persons suffer from the bites.

Certain assassin-bugs (Fig. 17) are of very definite medical interest in some localities. These bugs, called kissing-bugs because they usually take their blood meals from the lips, are important in the transmission of Chagas' disease. This disease, caused by a trypanosome (Trypanosoma cruzi), is primarily a childhood disease, but it not infrequently occurs in adults, and is very fatal. It occurs in South America, especially Brazil. The South American kissing-bug, Triatoma megista (syns. Conorhinus, Panstrongylus), is particularly important to preventive medicine since it serves as the principal vector of this disease. Closely related species occur in the southern United States, but no human cases of Chagas' disease have been reported from this country.

Siphonaptera (fleas). There are seven species of fleas of interest to the medical soldier: four because they are associated with human disease; three because they are commonly encountered and apt to be confused with those of medical importance. In this group also are to be found insects that both transmit disease and cause it directly.

The human flea (Pulex irritans) (Fig. 18) is found wherever man lives, but is widely distributed throughout the Western States, especially California. Like the bedbug, this flea lives in the cracks and crevices of the home, in the floors, rugs and bedding, emerging at night to attack the hosts. The human flea feeds readily on dogs, squirrels and other animals as well as on man.

The chigoe (Tunga penetrans) is the smallest flea known, and passes its life cycle as a fixed parasite of man and animals. The skin between the toes is most frequently attacked, producing irritation and swelling. This results



when the female fixes her mouthparts in the skin. The swelling encircles the entire insect except for a small opening at the hind end. It is through this opening that the chigoe is able to get air, and to lay her eggs which drop to the ground. Following this egg-laying, the flea shrivels up and dies. Secondary infections of the attacked sites is common. The chigoe is widely distributed in tropical America, and tropical Africa. It resembles the chicken flea very much in appearance (Fig. 18).

The tropical rat flea (Xenopsylla cheopis) (Fig. 18) is the most important vector of disease, particularly of bubonic plague. This flea is widely distributed in tropical regions throughout the world, and as a rule is not found in colder climates. It is distributed in the western and southern parts of the United States. Although primarily a parasite of the rat, during an epidemic it transmits plague from rat to rat, rat to man, and man to man.

The temperate zone rat flea (Ceratophyllus fasciatus) (Fig. 18) is another species of medical importance. It, too, maintains plague among rats and transmits the disease to man. It is world-wide in distribution, but for the most part is confined to the temperate zones.

The dog and cat fleas (Ctenocephalus canis and C. felis) (Fig. 18) are world-wide in distribution. The dog flea is widely distributed throughout the temperate climates of the United States and is the dominant ectoparasite of domestic pets, especially dogs and cats. The cat flea on the other hand, is more prevalent in warm climates, but may also be found in temperate regions. The cat flea has a wider range of hosts, but both species may infest man, rats and other mammals. These two species may be found in enormous numbers in homes where cats and dogs are kept as pets and allowed to sleep in the house.

The mouse flea (Leptosylla segnis) is a common ectoparasite of mice and rats in the Eastern Hemisphere but is also widely distributed in the Americas. This species is important in transmitting plague from rat to rat.

The chicken flea (Echidnophaga gallinacea) (Fig. 18) is of interest because it is commonly encountered and may be confused with more important species. It is very similar in appearance to the chigoe.

The most important human disease transmitted by fleas is plague, that age-old destroyer of mankind, caused by a bacillus (Pasturella pestis). Of all the fleas known to transmit this disease to man the Tropical Rat Flea is by far the most important. The Temperate Zone Rat Flea is able to transmit this disease but does not have much opportunity to do so since plague is somewhat limited to tropical countries. The Human Flea is considered important in transmitting plague among small animals and in times of epidemic may transmit the disease to man. Although other fleas may serve in this respect during epidemics, they are of very minor importance.

Murine or endemic typhus, a mild infection with Rickettsia prowazeki var. mooseri, is also transmitted by fleas. This infection, although not very common, is found somewhat frequently along the South Atlantic and Gulf Coasts. Although this disease may be transmitted from man to man by human body lice, it is commonly transmitted from rat to man by the Tropical Rat Flea and the Temperate Zone Rat Fleas.

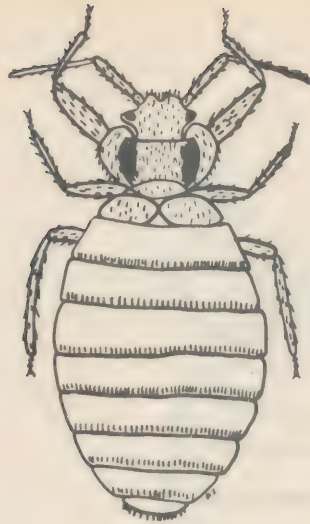


Fig.16. Bedbug (Heteroptera).

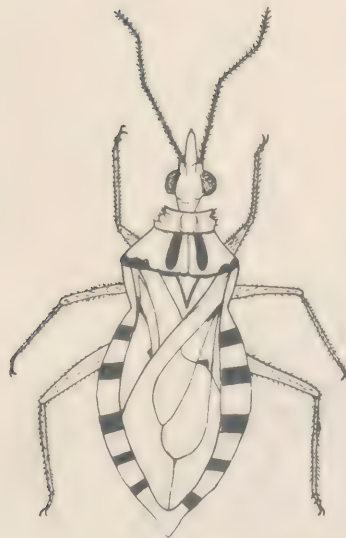


Fig.17. Kissing-bug (Heteroptera)







Fig.16. Bedbug (Heteroptera).



Fig.17. Kissing-bug (Heteroptera)

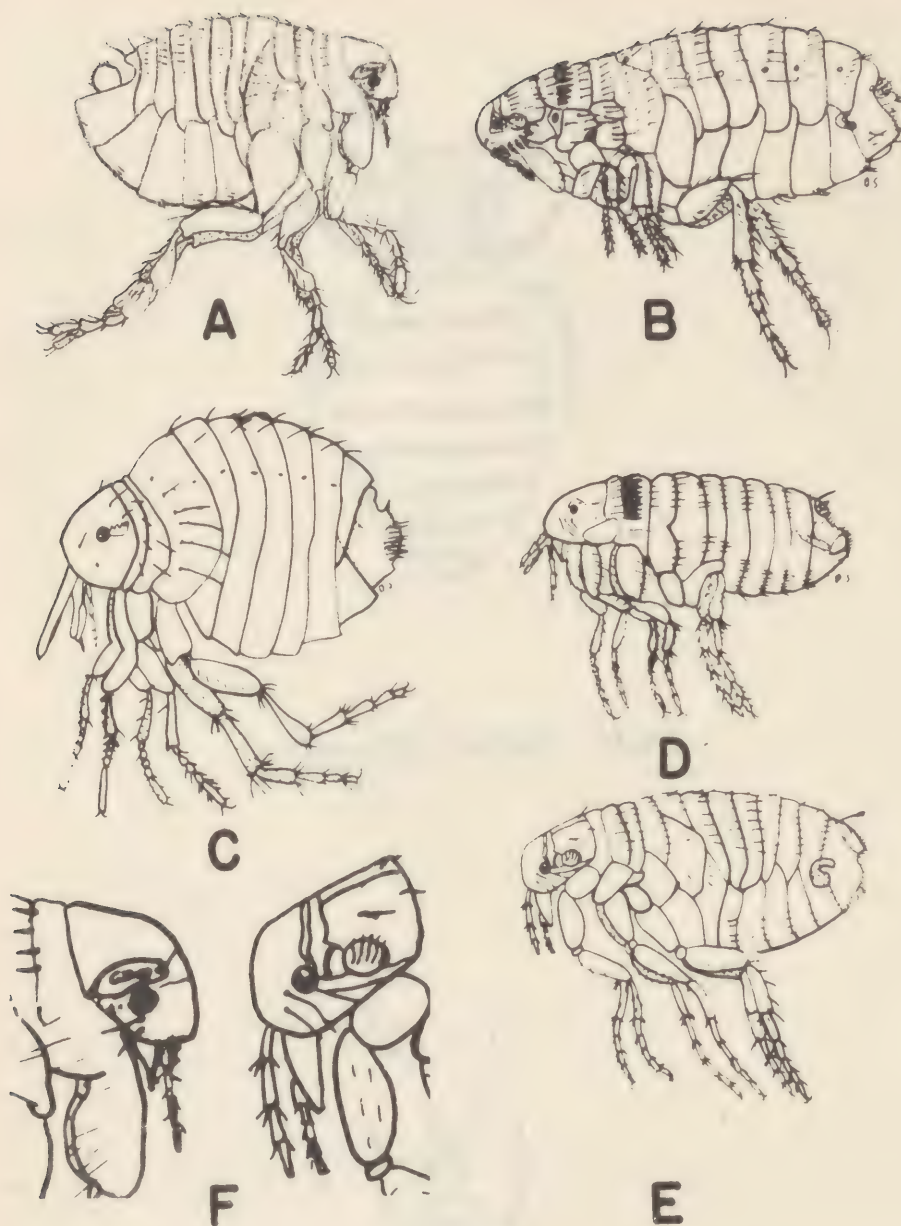


Fig. 18. Fleas (Siphonaptera). A, Human flea (Pulex irritans); B, Dog flea (Ctenocephalus canis); C, Chicken flea (Echidnophaga gallinacea); D, Temperate zone rat flea (Ceratophyllus fasciatus); E, Tropical rat flea (Xenopsylla cheopis); F, Heads of human flea (left) and tropical rat flea (right) showing arrangement of stout bristles in relation to eyes. (Note that in human flea a stout bristle is directly below the eye, whereas in the tropical rat flea it is in front of the eye.)

Some fleas, particularly the dog fleas, cat fleas, and human fleas, serve as intermediate hosts in certain helminth infections.

#### HANDLING AND SHIPMENT OF SPECIMENS

From time to time the medical soldier may have occasion to send specimens to an entomological center for identification. The Army Medical Museum at Washington, D. C. is closely associated with this work, but if the technician prefers to send his specimens to a more conveniently located center, the various corps area laboratories are available. For the most part, however, the technician should make a sincere effort to identify the specimen himself. As mentioned previously, this manual supplemented by various standard texts will enable the worker to determine whether the specimen is of medical importance, or at least, if it belongs to a medically important group.

Whenever it is necessary to ship specimens, they should be packed loosely in a small box between strips or sheets of lens paper or soft toilet tissue. They should not under any condition be packed in cotton because this material clings to the specimen and makes manipulation difficult. All packages containing these forms should be marked "fragile".



27  
21

# KEY FOR DETERMINING SPECIES OF ADULT FEMALE MOSQUITOES

- Wings spotted, palpi almost as long as the proboscis----- 1  
Wings unspotted, palpi much shorter than the proboscis----- 4
1. Palpi ringed and tipped with white----- 2  
Palpi uniformly dark brown----- 3
  2. Sixth longitudinal vein with three black spots--A. crucians  
Front margin of wing with three light spots, sixth (6th)  
longitudinal vein basally pale, apically black--A. pseudopunctipennis
  3. Prominent white spot on front wing margin. 6th  
longitudinal vein white with black ends; median grey thoracic  
stripe-----A. punctipennis  
Four dark spots on wing. 6th longitudinal vein wholly  
dark scaled, no thoracic stripe----- A. quadrimaculatus
  4. Legs banded or marked with white----- 5  
Legs unbanded, uniform in color----- 11
  5. Proboscis centrally ringed with white----- 6  
Proboscis unbanded----- 8
  6. Abdomen with dorsal longitudinal stripe, sides of thorax  
white below black stripe-----Aedes sollicitans  
Abdomen without dorsal stripe----- 7
  7. Large blackish species, tibia white spotted, femur white  
banded near tip, abdomen unbanded-----Psorophora columbiae  
Small blackish species, tibia and femur unmarked, Abdomen  
banded with white at bases of segments--Aedes taeniorhynchus
  8. Thorax marked with lines of stripes----- 9  
Thorax unmarked----- 10
  9. Large yellowish species, with broad yellow median thoracic  
stripe; legs heavily fringed with upright scales--Psorophora ciliata  
Small black species; thorax marked with white lyre shaped  
design; palpi white tipped; abdominal segments with lateral  
white spots-----Aedes aegypti
  10. White markings of legs narrow; abdominal bands nearly divided  
in the middle-----Aedes vexans  
Large purplish species, with two terminal joints and apex of  
middle joint of hind tarsi white-----Psorophora sayi
  11. Abdominal segments banded with white at the apex  
only----- Culex testaceus  
Abdominal segments banded with white at base only----- 12
  12. Under side of abdomen with unbroken black bands--Culex erraticus  
Under side of abdomen not completely banded----- 13
  13. Upper side with narrow white or yellowish basal bands--- 14  
Abdomen unbanded dorsally, lateral spots of the abdomen not  
visable dorsally -----Culex salinarius
  14. Band on second abdominal segment somewhat -  
triangular----- 15
  15. Abdominal bands joined to the lateral spots-----Culex pipiens  
Abdominal bands separated from the lateral spots--Culex quinquefasciatus





## Notes on the Dissection of the Adult Mosquito for Study

### 1. To mount the legs:

- (1) With a pair of sharp pointed scissors cut off one of the fore, middle, and hind pairs of legs immediately above the articulation of the tibia and the tarsus.
- (2) Place the specimens in absolute methyl alcohol and let stand for about ten minutes.
- (3) Remove the alcohol, add xylene, and let stand for ten minutes to clear.
- (4) Now carefully transfer the specimens to the middle of a clean plain slide, with small forceps or a large bore pipette.
- (5) Adjust the specimens in parallel arrangement with the middle leg in position between the fore and hind legs, preferably with the dorsal side down. The tarsal segment of the hind leg is markedly longer than that of the anterior legs, and will, therefore, aid in a rapid identification. A safe method that will insure proper final arrangement is to initiate the arrangement at the beginning of the process and then follow it throughout.
- (6) With a clean filter paper remove the excess xylene, add a drop or two of balsam on each leg, place the slide in a clean petri dish, cover just enough to preserve the slide from dust, and set aside for several hours or overnight to allow the balsam to settle firmly, insuring the permanent attachment of the specimens in position.

- (7) As soon as the specimens have become firmly attached to the surface of the slide, add another small portion of balsam over the specimens, place a drop of balsam in the center of a cover slip and invert over the specimens.
- (8) Return the specimens to the petri dish, cover, and set aside for several hours or overnight.
- (9) With a small piece of gauze and xylene, remove the surplus balsam from around the cover slide, label and study.
- (10) In many mosquitoes, the fore and middle pairs of legs bear fairly large claws at the tips of the fifth metatarsal segment. These claws may have simple structures, or they may be toothed in various ways which may give aid in classification. (See Illustrations Numbers 18, 19, and 20).

## 2. To mount the wings:

- (1) With sharp pointed scissors, cut off the wings as close to the thorax as possible, and place them in the concavity of slide.
- (2) Fill the concavity of the slide with absolute alcohol, and let stand for several minutes to remove the air from the lumina of the veins. It is best to place the slide into a petri dish and cover.
- (3) Remove the alcohol very carefully with a pipette, and then dry the slide thoroughly with a piece of filter paper.
- (4) Fill the concavity of the slide with hot carbol-fuchsin, place into a saturated petri dish, cover with lid, place in hot chamber or incubator, and let stand for one hour or more.

- (5) Remove the stain very carefully with a capillary pipette, and fill the concavity of the slide with seventy per cent alcohol, let stand for about three minutes, then remove and add fresh seventy per cent alcohol. Repeat this process until the wing cells lose their color while the veins remain deeply stained the decolorization under the microscope.
- (6) Remove the seventy per cent alcohol, add absolute alcohol, place in saturated petri dish, cover, and set aside for about five minutes. Repeat this process once.
- (7) Remove the alcohol, add xylene, and let stand about ten minutes.
- (8) With a capillary pipette and xylene, very carefully wash the wings into the middle of a clean plain slide. Arrange the wings; remove the xylene, add a drop or two of balsam, and cover with a cover slide.
- (9) Set aside to dry, remove the surplus balsam, and label.

(See Illustration Number 21 for study of wing)

### 3. To mount the head and its appendages:

To insure that the head and appendages are not distorted in any way, it is necessary to employ a careful technique in this process. It will be safer to use the concave slide throughout.

- (1)(2) Remove the head of each of a freshly anesthetized male and female mosquito, by carefully through the neck, passing the sharp point of the scissors between the head and thorax. This part of the technique should be executed on a broad plain glass slide.
- (2) Carefully transfer the specimens to the concavity of a slide. Fill the concavity with ten per cent sodium hydroxide solution.
- (3) Place the slide into a saturated petri dish, cover with lid and in warm chamber or incubator for several hours or overnight. This will dissolve the pigment and soft tissues.
- (4) Remove the sodium hydroxide solution, add distilled water, and let stand for several minutes to wash out the hydroxide. Repeat this washing process three or four times.



- (5) Add absolute alcohol, and let stand for about five minutes; remove, and add more alcohol. Repeat the process twice more.
  - (6) Remove alcohol and add xylene and let stand for about five minutes. Renew the xylene and let stand for another five minutes.
  - (7) Remove the xylene and place on balsam to fill the concavity of the slide. Place on cover slide. Set aside to dry. Label and study.
4. ( To mount thorax for study of lateral aspect: -
- (1) Carefully remove the head, wings, and legs and abdomen. Follow the same technique as in 3 above, finally mounting the specimen with the lateral aspect uppermost.
5. To mount the genitalia of the male and female:
- (1) Cut off the tip of the abdomen at the sixth segment. Follow the same technique as in 3 above, finally mounting the specimens with their ventral aspect uppermost.
6. To remove the stomach, Malpighian tubes, hind intestine, and the reproductive organs:
- (1) Having anesthetised the mosquito, empty it onto a sheet of white paper and remove the wings and legs.
  - (2) Place a drop of one per cent sodium chloride solution upon a clean slide.
  - (3) With a pair of clean forceps, momentarily dip the mosquito into seventy per cent alcohol, and then immediately place in the solution on the slide. The specimen will partially submerge.
  - (4) Now with the dissecting needles, adjust the body of the mosquito in the salt solution, so that it lies on its side horizontally in the field of the objectives of the microscope, (See Illustration Number 21 G), the tip of the abdomen in position on the right-hand side of the field of vision.

- (5) With the dissecting needles, held one in each hand, steady the body of the mosquito by placing the needle held in the left-hand across the thorax and make two tiny cuts in the integument of the abdomen, one on each side, between the junctions of the sixth and seventh abdominal segments, with the point of the needle held in the right hand. (See Illustration Number 21 G).
- (6) Now with the needle which is held in the left-hand, sever the abdomen from the thorax as close as possible to the posterior aspect of the thorax. Place the head and thorax in a drop of salt solution on another slide.
- (7) Arrange the abdomen to the center of the field, and place the sloped point of the left-handed needle so that it will be in position marked B in Illustration Number 21 G. At the same time, place the sloped point of the right-hand needle on the seventh abdominal segment just behind the two small cuts which were previously made in the integument. (See Illustration Number 21 G).
- (8) Hold the left-hand needle steady, and using a firm but light traction on the right-hand needle, pull the seventh segment and the tip of the abdomen slowly and steadily from the anterior segments.
- (9) At first, a small section of the hind intestine will be seen immediately <sup>as</sup> the integument ruptures at the cuts. Continue to exert firm and steady traction, and, as the segments are pulled away from each other, one or two folds of the Malpighian tubes will appear along with the lower portion of the ovaries if the specimen is a female. As soon as the organs are visible, cease the progressive pulling action, but maintain the tension, this will enable the abdominal walls to gradually expand, and allow time for the fine branches of the tracheae to break under the continued strain, decreasing the risk of breaking the organs.

- (10) As soon as the tension has decreased through the expansion of the abdominal walls, renew the pulling action of the needle, and by intermitten pulling accompanied with short intervals of rest while the tension is maintained, the stomach along with the other organs will be drawn out of the abdomen.
- (11) Wash the organs into the concavity of a slide, arrange in order (See Illustration Number 21 H and J), in the concavity of the slide.
- (12) Remove the salt solution, fill the concavity of the slide with Carnoy's solution, place slide into a saturated petri dish, close and set aside for about thirty minutes.
- (13) Remove the Carnoy's solution, carefully make two or three washings with absolute alcohol, at about ten minute intervals. Now wash in thirt per cent alcohol.
- (14) Remove the thirty per cent alcohol, add Borax Carmine for staining over night, in a saturated petri dish.
- (15) Remove the stain and wash several times in acidulated thirty per cent alcohol, until the specimen has a pink color. (Acidulated alcohol: one drop of acetic acid to ten cc of the thirty per cent alcohol).
- (16) Remove the acidulated alcohol, add seventy per cent alcohol and let stand for about ten minutes. Remove the alcohol, and repeat the washings for two or three times, at five minute intervals.
- (17) Remove the seventy per cent alcohol, and wash twice in absolute alcohol at ten minute interval.



9

(18) Clear in xylene for about ten minutes, using two washings.

(19) Remove the xylene, add balsam and cover the specimen with a cover glass. Set aside to dry.

(20) Remove the surplus balsam from around the cover glass and label.

7. To remove the salivary glands, anterior portion of the esophagus, and food reservoirs:

- (1) Using the head and thorax of the mosquito left over from 6 above, adjust the portion of the body so that the head is in position on the right-hand side of the microscope.
- (2) The salivary glands are embedded in the thorax at a point a little above the attachment of the middle pair of legs (See Illustration Number 21 J), therefore guard against injury to this area.
- (3) Carefully insert the left-hand needle into the posterior latero-dorsal angle of the thorax, until the point of the needle strikes the surface of the glass slide. This will steady the thorax.
- (4) Avoiding the neck, pass the point of the right-hand needle obliquely through the back of the head, so that the needle point will rest against the posterior margin of one of the compound eyes.
- (5) With a slow forward and downward traction with the right-hand needle, drag the head away from the thorax. The glands and other organs will be pulled out of the thorax (See Illustration Number 21 K for a picture of the glands).

(6) Now wash the glands into the concavity of a slide, arrange in order, and then follow as from (12) under 6 above.

#### 8. For infected mosquitoes:

In infected mosquitoes the technique for dissecting is the same as for dissecting normal mosquitoes. However the salivary glands are usually stained with Wright's stain, or with Geimsa's and the stomach with some of the hematoxylin stains.

#### \* Carnoy's Solution:

Absolute alcohol	60 parts
Chloroform	30 parts
Acetic acid	10 parts

(This is a very powerful fixative and nearly all stains follow it easily. When using Wright's stain it will not be necessary to fix in Carnoy's).

Reproduced and Modified from Figure 47, Plate 314,  
Hemite Survey by Macrator, 1923.



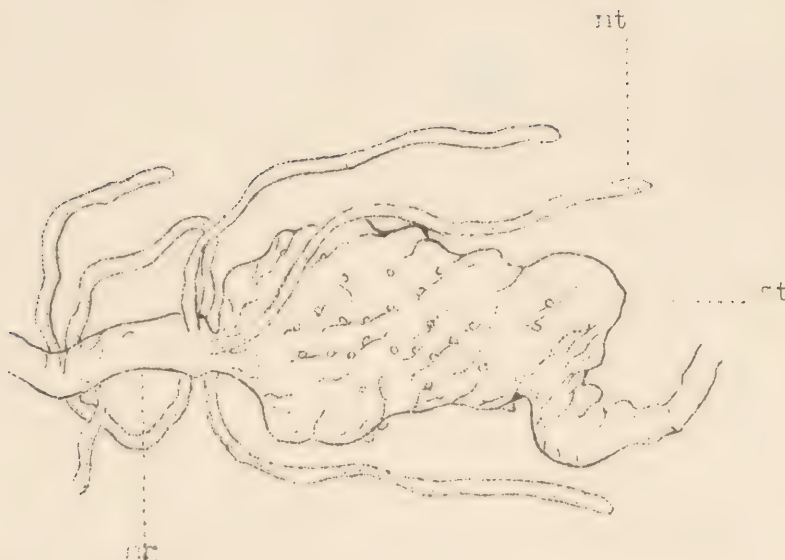
A.J. Babalonis

Illustration Number 21 G





Re-produced and modified from Figure 48, page 216, Mosquito Stomach,  
November, 1908.



Mosquito Stomach.

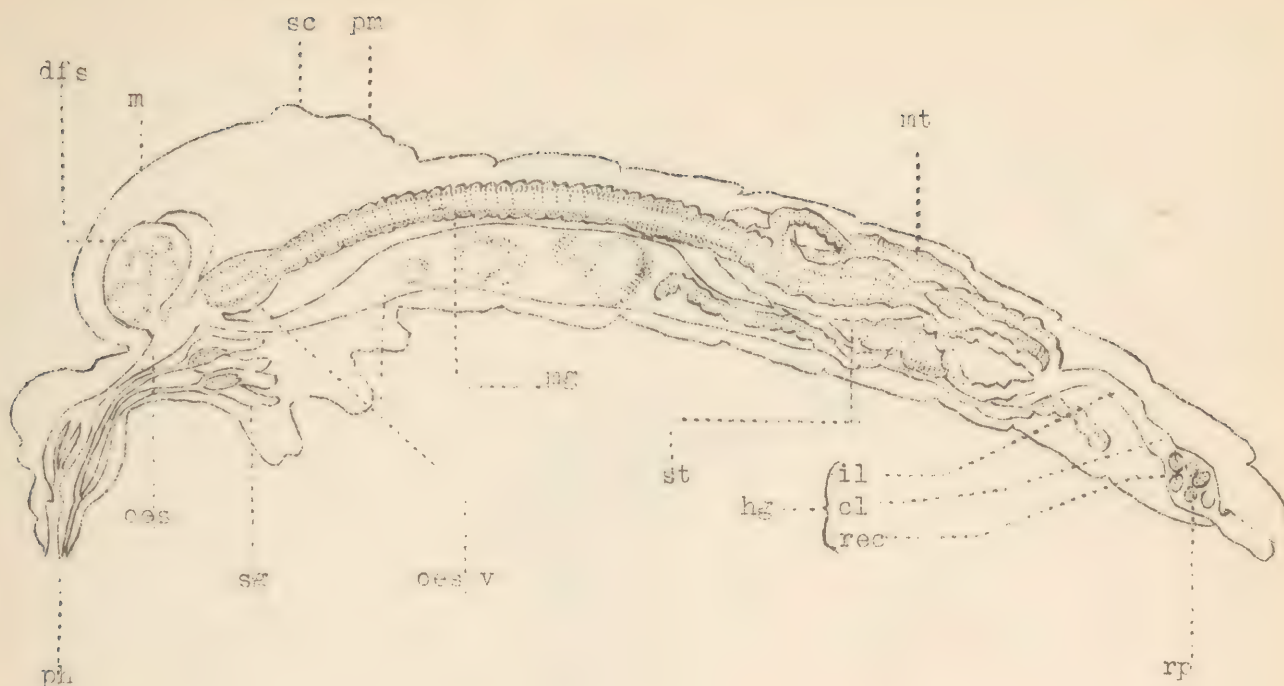
nt = midgut  
nt = malpighian tubules  
st = stomach

A.J. Bablonis

Illustration Number 21 H







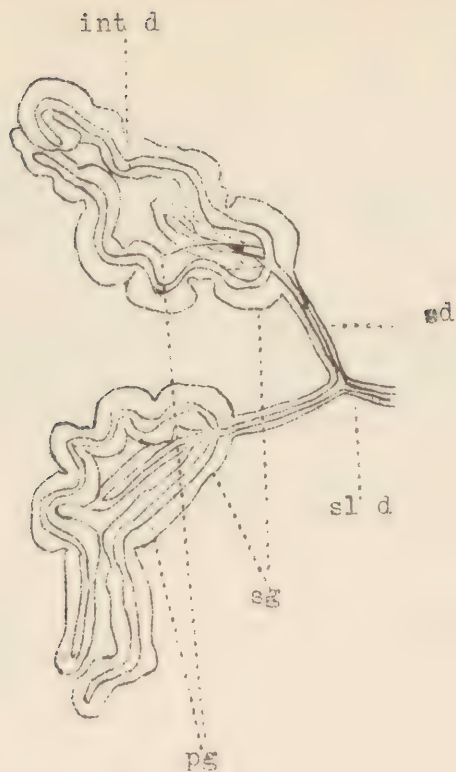
Internal Anatomy of a Female Anopheles Mosquito

m=	Mesonotum	vfs=	Ventral Food Reservoir
dfs=	Dorsal Food Reservoirs	mg=	Midgut
sc=	Scutellum	mt=	Malpighian tubule
pn=	Postnotum	st=	Stomach
ph=	Pharynx	hg=	Hindgut
oes=	Oesophagus	il=	Ileum
sg=	Salivary Glands	cl=	Colon
oes v=	Oesophageal Valve	rec=	Rectum
	rp= Rectal Papillae		

Illustration Number 21 I

*C. J. Babalonia*





Two Salivary Glands of a Female Mosquito

sl d- Salivary duct  
 sd- Side duct  
 int d- Intra duct  
 sg- Salivary gland  
 pg- Poison gland

Illustration Number: 21 K

*A. J. Bateman*





S. M. D. T. - Laboratory Section

1st Day - 9 to 12 A.M.

Lecture -

1. Classification of bacteria
2. Selection of culture media
3. Bacteria commonly found in pus and exudates  
(a) Genus Staphylococcus

Laboratory -

1. Cultures of Staphylococcus aureus and S. albus will be supplied.  
(a) Streak each on (1) blood plate and (2) plain agar plate.  
(b) Examine fresh and Gram-stained films of each microscopically.
2. Examine any available clinical material (pus from boil, or throat swab):  
(a) Prepare film on slide, fix, stain and examine.  
(b) Inoculate a blood agar plate and a plain agar plate.

Study Assignment:

Simmon's pp. 604-605  
Simmon's pp. 613-614  
Simmon's pp. 660-662

2nd Day - 9 to 12 A.M.

Lecture -

General laboratory methods for the culturing and identification of Gram-positive cocci.

Laboratory -

1. Examine plates streaked yesterday.  
(a) Note color, size, shape and consistency of colonies.  
(b) Pick colonies and examine organisms microscopically.  
(c) Set agar plates streaked with Staphylococcus aside at room temperature for pigment formation.  
(d) Select well isolated colonies of different types (Staph. and Strep.) and transfer to portion of blood plate.
2. Examine wet and stained preparations of Goffkya tetragena and Sarcina lutea. Study morphology.
3. Cultures of St. pyogenes and St. salivarius will be supplied.  
With each inoculate blood plate and tube of infusion broth.

Study Assignment -

Simmon's pp. 662-664.

3rd Day - 9 to 12 A.M.

Lecture -

Genus Streptococcus.

Laboratory -

1. Examine pure cultures of St. pyogenes and St. salivarius.
  - (a) Blood plates - note colony formation and type of hemolysis.
  - (b) Broth cultures - note type of growth.
  - (c) Make smears of organisms from plates and from broth. Stain by Gram's method and examine.
2. Examine transfer plate from clinical material.
  - (a) Determine purity of culture.
  - (b) Classify the organisms.

Study Assignment -

Simmon's pp. 643 - 650.

4th Day - 9 to 12 A.M.

Lecture -

Technic for making blood cultures.

Laboratory -

1. Sections will make cultures of blood specimens supplied by Instructor.

Study Assignment -

Simmon's pp. 606 - 607

Simmon's pp. 705.

WRITTEN EXAMINATION.

5th Day - 9 to 12 A.M.

Lecture -

1. D. pneumoniae.
2. Technique of rapid identification.

Laboratory -

1. Examination of clinical material - sputum from pneumonia patient.
  - (a) Determine type by Neufeld's "Quelling" reaction.
  - (b) Inoculate a blood agar plate.
  - (c) Make smear on slide, fix, stain and examine.
2. Each section will inoculate two mice with pneumococcic sputum.
3. Using culture of D. pneumoniae supplied by instructor, inoculate blood agar plate and tube of  $\text{Ca CO}_3$  broth.



Study Assignments -

Simmon's pp. 636 - 643.

6th Day - 9 to 12 A. M.

Laboratory -

1. Study the pure cultures of D.pneumoniae prepared yesterday.
  - a. Set up bile solubility test.
  - b. Inoculate tube of inulin broth.
  - c. Study colonies on plate culture.
  - d. Examine stained smears.
2. Examine blood plate culture for D. pneumoniae type colonies.
3. Autopsy mice, collect peritoneal washings and determine type of D.pneumoniae present.

7th Day - 9 to 12 A.M.

Lecture -

1. Genus Neisseria.
2. Neisseria gonorrhoeae.

Laboratory -

1. Demonstration of technic for culturing N.gonorrhoeae.
2. Each student will be supplied with cultures of N.intracellularis; N.gonorrhoeae; N.catarrhalis and N.flava.
  - a. With N.intracellularis culture inoculate (1) two blood agar plates; (2) one blood agar slant; (3)  $\frac{1}{4}$  of infusion agar plate and (4) Loeffler's medium.
  - b. With N.gonorrhoeae; N.catarrhalis and N.flava cultures, plant each on:
    - (1)  $\frac{1}{3}$  of two blood agar plates.
    - (2) 1 blood agar slant.
    - (3)  $\frac{1}{4}$  infusion agar plate.
    - (4) 1 tube of Loeffler's medium.
  - c. Examine Gram-stained films of each culture.
3. With above cultures of N.gonorrhoeae and N.intracellularis, inoculate set of serum broth sugars (glucose, maltose, saccharose and levulose).

Note -

Incubate 1 each of the blood agar plates under 2 a and 2 b at room temperature and the other cultures at 37°C.

Study Assignment -

Simmon's pp. 651 - 653.  
Difco Manual pp. 92 - 96.

8th Day - 9 to 12 A.M.

Laboratory -

1. Smears from cases of acute and chronic gonorrhoea will be supplied for study.
2. Examine Neisseria cultures on agar for growth.
3. Examine Neisseria group cultures incubated at room temperature for growth.
4. Examine growth of Neisseria group organisms on blood plates inoculated yesterday.
  - a. Study colony formation by reflected and transmitted light.
  - b. Examine Gram-stained smears of each organism.
5. Record reactions in sugar tube cultures.

9th Day - 9 to 12 A.M.

Lecture -

Neisseria intracellularis.

Laboratory -

1. Make 48 hour readings of Neisseria cultures for growth, sugar fermentation, colony formation and pigment production.
2. Practice the presumptive slide agglutination test for meningitis, using N.intracellularis and N.catarrhalis cultures.
3. Designated students from each section will set up microscopic test tube agglutination tests against polyvalent and type specific meningococcus antisera, using N.intracellularis and N.catarrhalis antigens.

Study Assignment -

Simmon's pp. 653 - 660.

10th Day - 9 to 12 A.M.

Laboratory -

1. Read agglutination tests set up yesterday and record results.
2. A series of unknown slides will be supplied. Examine for G.C.

Written Examination.

11th day - 9 to 12 A.M.

Lecture -

1. Bacteriological Examination of water.
  - a. Apparatus required.
  - b. Special culture media used.
  - c. Collection of samples.
  - d. Total bacterial count.
  - e. Tests for presence of coli-aerogenes group (1st and 2nd days)

Laboratory -

1. Each student will set up two complete tests of water.
  - a. Water sample #1 - Water from a laboratory faucet.
  - b. Water sample #2 - Water from Rock Creek.
2. Inoculate 3 infusion agar slants with culture of Staph. aureus for preparation of an autogenous vaccine (Wednesday).

Study Assignment -

Standard Method of Water Analysis pp. 196 - 208 and 210 - 211.

12th Day - 9 to 12 A.M.

Lecture -

1. Bacteriological examination of water - the completed test for presence of coli-aerogenes group.
2. Bacteriological control of swimming pools.

Laboratory -

1. Count agar plates from water specimens Nos. 1 and 2:
  - a. using Quebec colony counter
  - b. using colony counting plate
2. Observe lactose tubes from water specimens, Consult with instructor as to further procedure.
3. Make heavy saline suspension from 3 slants of Staph. aureus. Transfer to ampule and seal. Heat at 58-60° C. for one hour. Store in icebox.

Study Assignment -

Standard Method of Water Analysis pp. 209, 212 - 217 and 286 - 288.

13th Day - 9 to 12 A.M.

Lecture -

1. Preparation of autogenous vaccines.



Laboratory -

1. Proceed with examination of water specimens. Consult instructor.
2. Open vial of killed culture of Staph. aureus prepared yesterday:
  - a. Transfer aseptically to sterile test tube.
  - b. Culture for sterility:
    - (1) Plain agar pour plate.
    - (2) Freshly boiled glucose agar stab.
  - c. Standardize vaccine by Nephelometer Method. Dilute as indicated, add 0.5% phenol, bottle 25 cc, and label.

Study Assignment -

Simmon's pp. 592 - 596,

14th Day - 9 - 12 A.M.

Lecture -

1. Bacteriological examination of milk.

Laboratory -

1. Samples of milk will be supplied. Each student will inoculate plates for making total colony count.
2. Continue examination of water specimens.
3. Observe vaccine cultures for sterility.

Study Assignment -

Simmon's pp. 766 - 771.

15th Day - 9 to 12 A.M.

Laboratory -

1. Make count of colonies on milk plates.
2. Complete examination of water specimens. (Save any coli-aerogenes group culture for use.)
3. Make final observation of cultures for sterility of auto-genous vaccine.

Written Examination.

16th Day - 9 to 12 A.M.

Lecture -

1. The Gram negative enteric bacilli
  - a. The coli-aerogenes group
    - (1) Genus - Escherichia
    - (2) Genus - Aerobacter
    - (3) Genus - Klebsiella
  - b. The proteus group
    - (1) Genus - Proteus
2. Biochemical tests for identifying coli-aerogenes group.

Laboratory -

1. Pure cultures of E. coli and A. aerogenes will be supplied.
  - a. Examine the Gram stained smears of each.
  - b. Inoculate each culture upon the following media:
    - (1) E.M.B. plate
    - (2) Russel's double sugar medium
    - (3) Dunham peptone
    - (4) Clark and Lub's medium
    - (5) Citrate agar
2. Pure cultures of E. typhosa, Salmonella schottmulleri (Para B), and Salmonella paratyphi (Para A) will be supplied.
  - a. Inoculate each culture upon the following media:
    - (1) E.M.B. plate
    - (2) Russel's double sugar
    - (3) Five sugars - lactose, dextrose, saccharose, mannite and xylose.

Study Assignment -

Simmon's pp. 683 - 685, 692 - 707  
Standard Method of Water Analysis pp. 269 - 272.

17th Day - 9 to 12 A.M.

Lecture -

1. The pathogenic Gram-negative enteric bacilli
  - a. Day by day procedures for identifying typhoid-dysentery group organisms.
  - b. Genus Eberthella and Salmonella
    - (1) Cultural methods
    - (2) Serological methods (Widal macroscopic test tube agglutination, etc.)
    - (3) Maintenance of records (Biochemical reactions and agglutinations)
    - (4) Precautions in handling cultures.

## Laboratory -

1. Examine cultures of *E. coli* and *A. aerogenes*
  - a. Study appearance of colonies on E.M.B. plates.
  - b. Study and record type of reaction on RDS tubes and citrate tubes.
  - c. Re-inoculate Dunham peptone tubes and Clark and Lub's tubes for four days.
2. Examine cultures of *E. Typhosa*, Para A and Para B.
  - a. Study appearance of colonies on EMB plates, make motility tests.
  - b. Study and record type of reaction on RDS tubes.
  - c. Record reaction on sugar tubes and re-inoculate for five days recording reaction daily.
3. Inoculate two Pa slants with colonies picked from EMB plates (*E. typhosa*, Para A and Para B)
4. A specimen of feces containing an unknown member of *Salmonella* - *Eberthella* group will be supplied. Plate each on EMB plates.

## Study Assignment -

Simmon's pp. 707 - 710, 623 - 629.

18th Day - 9 to 12 A.M.

## Lecture -

1. The pathogenic Gram-negative enteric bacilli
  - a. Day by day procedures for identifying typhosa dysentery group organisms.
  - b. Genus - *Shigella*
    - (1) Cultural methods
    - (2) Serological methods
    - (3) Maintenance of records (Biochemical reactions and agglutinations)
    - (4) Precautions in handling materials.

## Laboratory -

- A. Examine EMB plates streaked with feces yesterday
  1. from each specimen mark suspicious colonies and transfer each to RDS tubes and PA slants.
- B. Pure cultures of *S. Dysenteriae* and *S. Paradyenteriae* will be supplied.
  1. Inoculate each culture upon the following media:
    - a. EMB plate,
    - b. Russel's Double Sugar
    - c. Five sugars (Lactose, Dextrose, Saccharose, Mannite and Xylose)



- C. Prepare antigen from PA slants inoculated with *E. typhosa*, Para A and Para B.
1. Set up macroscopic test tube agglutination test.
  2. Record sugar reactions and re-incubate sugar.

Study Assignment -

Simmon's pp. 697 - 710, 623 - 629.

19th Day - 9 to 12 A.M.

Lecture -

1. The pathogenic enteric bacilli (Gram-negative)  
Review of day by day procedures for identifying typhoid - dysentery group organisms.
  - a. Clinical materials
  - b. Preparation and shipment of specimens
  - c. Special media (preservatives)
  - d. Precautions in handling materials
  - e. Requisitions for anti sera and antigens.

Laboratory -

1. Read the agglutination test set up yesterday.
2. Examine cultures of *S. dysenteriae* and *S. para-dysenteriae*
  - a. Study appearance of colonies on EMB plates
  - b. Study and record types of reaction on RDS tubes
  - c. Record reactions of sugars
  - d. Perform motility tests
  - e. Inoculate 2 PA slants with colonies picked from EMB plates.
3. Prepare antigen from PA slants from suspicious colonies.
  - a. Set up macroscopic test tube agglutination test.
  - b. Record sugar reactions and re-incubate sugars.

Study Assignment -

Simmon's pp. 707 - 710, 623 - 629, 596 - 602.

20th Day - 9 to 12 A.M.

Lecture -

The pathogenic Gram- negative enteric bacilli

- a. Special procedures for identifying typhoid dysentery group organisms.
- b. Anti sera and antigens
- c. Review

Laboratory -

1. Read and record agglutination tests set up yesterday.

2. A specimen of feces containing an unknown member of *Shigella* group will be supplied.
3. Plate each on an EMB plate
4. Prepare antigen from two PA slants inoculated yesterday with *S. dysenteriae* and *S. para-dysenteriae*
5. Set up macroscopic agglutination test.
6. Record sugar reactions and re-incubate sugar

Study Assignments -

Simmon's pp. 697 - 710, 623 - 629.

21st Day - 9 to 12 A.M.

Lecture -

Bacterial Food poisoning.

Laboratory -

1. Read agglutination test set up yesterday and record.
2. Examine EMB plates streaked with feces yesterday.
  - a. Study appearance of suspicious colonies on EMB plates.
  - b. Inoculate RDS tubes and PA slants with suspicious colonies.
3. Set up agglutination tests with unknown antiserum, supplied plus *S. paratyphi* and *S. schottmulleri* antigens.
4. Record sugar reactions and re-incubate sugars.

Study Assignment -

Simmon's pp. 763 - 765, 623 - 629

22nd Day - 9 to 12 A.M.

Laboratory -

1. Read and record agglutination test set up yesterday
2. Prepare antigens from PA slants made yesterday
3. Record sugar reactions and re-incubate sugars. Complete cards on sugar Biochemical reactions and agglutinations and turn in to instructor.

Written Examination:

23rd Day - 9 to 12 A.M.

Lecture -

1. *Vibrio comma*.

Laboratory -

1. Perform methyl red test on typhoid-dysentery culture inoculated 6/9. Also make final observations on other cultures.
2. Pure cultures of V. comma and a non-pathogenic Vibrio will be supplied.
  - a. Inoculate each (1) into Dunham's peptone and (2) on infusion agar plate.
  - b. Examine preparations for (1) motility, (2) morphology of organisms, and (3) Gram-staining properties.
3. Streak Hemophilus influenzae and H. pertussis cultures on blood agar and on infusion agar plates.

Study assignment -

Simmon's pp. 664 - 669.

24th Day - 9 to 12 A.M.

Lecture -

Genus Brucella and other Parvobacteriaceae.

Laboratory -

1. Pure cultures of Vibrio:
  - a. Study type of colonies on plates.
  - b. Observe type of growth in peptone broth. Perform "Cholera Red" test.
2. Demonstration of cultures of P. tularensis. Handle carefully. Do not open.
3. Examine stained smears of Pasteurella from cultures and animal tissue.
4. Stain and examine smears of killed cultures of Brucella.
5. Set up agglutination test with Brucella and P. tularensis antigens.
6. Cultures of Hemophilus influenzae and H. pertussis. Study colony formation and morphology of organisms.

Study Assignment -

Simmon's pp. 671 - 683, 685 - 689, and 711 - 716.

25th Day - 9 to 12 A.M.

Lecture -

1. Sporulating bacteria.
  - a. Genus Bacillus.
2. Anaerobic Culture Methods.

Laboratory -

1. Read agglutination tests from yesterday.



2. Cultures of Bacillus subtilis and B. anthracis will be supplied for study.
3. Demonstration of anaerobic culture methods.
  - a. set up "Brown Jar" containing plate and tube cultures of Clostridium perfringens.

Study Assignment -

Simmon's pp. 717 - 720, 616 - 623.

26th Day - 9 to 12 A.M.

Lecture -

Sporulating bacteria.

- a. Genus Clostridium.

Laboratory -

1. Cultures of Cl. perfringens, Cl. histolyticum, Cl. parabolulinum and Cl. tetani will be supplied.
  - a. Test for motility.
  - b. Examine for presence and position of spores.
2. Open "Brown Jar" and examine cultures.

Study Assignment -

Simmon's pp. 720 - 735

27th Day - 9 to 12 A.M.

Lecture -

1. Spirochetes.
  - a. Treponema
  - b. Leptospira.
2. The "Dark-field method" of demonstrating bacteria.

Laboratory -

1. Practice in use of "Dark-field".
2. Demonstration of stained spirochaetes.

Study Assignment -

Simmon's pp. 611 - 612, 757-761.

28th Day - 9 to 12 A.M.

Lecture -

1. Spirochetes.
  - a. Borrelia - Vincent's Angina

Laboratory -

1. Demonstration of stained slides showing Borrelia.
2. Stain and examine films for Vincent's organisms.

Study Assignment -

Simmon's pp. 754 - 757.

Written Examination.

29th Day - 9 to 12 A.M.

Lecture -

Genus Corynebacterium.

- a. C. diphtheriae.
- b. Technique for Neisser's stain.

Laboratory -

1. Each student will be furnished with pure cultures grown for 18 to 24 hrs. on Loeffler's medium of C. diphtheriae, C. pseudodiphthericum and C. jeikeium.
  - a. Stain and examine smears of each by Grams' method and by Neisser's method.
  - b. With each, inoculate a set of following sugar media: (1) dextrose, (2) maltose; (3) saccharose; and (4) dextrin.
  - c. Streak each culture on section of: (1) blood agar plate, and (2) potassium tellurite agar plate.
  - d. Transfer each to tube of Loeffler's medium.

Study Assignment -

Simmon's pp. 745 - 750 and p. 563 (Neisser's stain)

30th Day - 9 to 12 A.M.

Lecture -

Virulence tests for C. diphtheriae.

Laboratory -

1. Examine all cultures made yesterday.
  - a. Note and record reactions in sugar media.
  - b. Study growth on plates for color changes and morphology of colonies.
  - c. Examine stained smears from cultures on Loeffler's media.
2. Several groups will be designated to do subcutaneous Virulence tests.

Study Assignment -

Simmon's pp. 749.

31st Day - 9 to 12 A.M.

Lecture -

Genus Mycobacterium.

- a. Technique of Zeihl-Neelsen staining method.
- b. Microscopic examination of clinical material.

Laboratory -

1. Several specimens of T.B. sputum will be supplied for practice in staining and recognizing acid-fast organisms. Each student will stain and examine at least six preparations.

Study Assignment -

Simmon's pp 738 - 743, 563 - 564 (Ziehl - Neelsen staining method).

32nd Day - 9 to 12 A.M.

Lecture -

1. Genus Mycobacterium (concluded).
  - a. Concentration of specimen.
  - b. Culturing and animal inoculations
  - c. M. leprae.

Laboratory -

1. Concentrate specimen of sputum by "Sodium hydroxide - alum flocculation method."
  - a. Prepare smear, stain and examine for T.B.
  - b. Make culture on Petroff's medium.
  - c. Students will be designated to inoculate guinea pigs.
2. Demonstration of M. leprae slides.
3. Demonstration of guinea pig showing tubercular lesions.
4. Demonstration of stock cultures of Mycobacteria.

Study Assignment -

Simmon's pp. 743 - 744, p. 589 (Petroff's medium).

33rd Day - 9 to 12 A.M.

Discussion and demonstration of yeasts and moulds.

Study Assignment -

Simmon's p. 590, ;;. 537 - 554.



34th Day - 9 to 12 A.M.

Yeasts and Moulds (concluded).

Written Examination.

(Daily work schedule to be prepared by Lt. Cebel.)

35th and 36th Days - 9 to 12 A.M.

Rickettsia and Virus Diseases.



Habitat: Common pathogenic forms; also frequently on skin and body orifices without invasive tendency. Some species are the specific cause of infectious diseases. A number of saprophytic species are commonly present in dairy products and elsewhere.

Characteristics: Gram positive cocci of medium size, in pairs or short chains, never in packets; grow best on blood or serum agar, aerobically, at 37° C., the 24 hour colony being small, circular, slightly raised, surrounded at times by zone of haemolysis. Killed at 55° C., in 30 minutes.

Haemolytic Group (Beta type) have clear zone of haemolysis around colony on blood agar.

Viridans Group (Alpha type) have greenish zone around colony on blood agar.

Non-haemolytic Group (Gamma type) have no area of haemolysis or green zone around colony.

Streptococcus pyogenes: Colonies have Beta zone of haemolysis 2 to 3 mm. wide. Grow in long chains. Found in man in acute inflammations including septicaemia, cellulitis, wound infections, middle ear or sinus disease or elsewhere. Tend to be more severe and generalized than Staph. aureus infections. The cause of scarlet fever, transmitted by nose, throat and skin contaminations from cases or carriers, and of erysipelas.

Streptococcus salivarius: (S. viridans) (S. mitior) is a parasite of the normal nose and throat, also encountered in dental abscesses, in endocarditis and in some blood cultures. Grow in short chains. This colony readily recognized on a blood agar plate by its greenish-zone of haemolysis. Usually not pathogenic for small animals. Distinguished from Diplococcus pneumoniae by inability to ferment inulin and by not being bile soluble.

Streptococcus lactis: Is non-pathogenic, occurs in milk and milk products and in mouth and intestinal tract of man. Colonies on blood plates produce no haemolysis or only trace of green.

Streptococcus faecalis: Is feebly pathogenic, found in feces of man and other animals. Sometimes found in inflammatory exudates and subacute endocarditis. No haemolysis on blood agar.

Identification:

1, Microscopic: Gram stain of direct or culture spreads will show Gram + cocci, singly, in pairs or in chains of varying length. The chain form is best seen in spreads made from liquid culture, or in liquid body fluids.



2. Culture: Blood agar plates at 37° for 24 hours will give the small colony type and form of haemolysis classifying roughly the species. For routine clinical work the examination is usually limited to the study of colonies on blood agar and the results reported as the case may be:

Streptococcus	Haemolytic
"	non-haemolytic
"	viridans

### GENUS STAPHYLOCOCCUS

Habitat: Common, potential or actual parasites, occurring on normal skin and body orifices, and in feces, therefore in dust, soils and as culture contaminants; frequently the cause of suppurative lesions in man.

Characteristics: Moderate size cocci, are in pairs or grape-like clusters; Gram-positive; grow freely, aerobically, on common culture media, giving in 24 hrs. at 37°C. medium size, low, convex, smooth, glistening colonies with an even edge; color of colony variable with species; some strains produce haemolysis on blood agar.

Staphylococcus aureus: Golden yellow colony; usually haemolytic; frequently found in boils, carbuncles and other skin lesions; sometimes in blood cultures in the event of septicaemia.

Staphylococcus albus: Porcelain-white colony; feebly pathogenic.

Staphylococcus citreus: Lemon-yellow colony. A non-pathogenic saprophyte.

### Identification:

1. Microscopic: Gram / staphylococci on direct or culture stained spread.
2. Culture: blood agar plate, 24 hrs. at 37° C. gives colony features of staphylococcus, species determined by color of colony. Note also presence or absence of haemolysis.

## DIPLOCOCCUS PNEUMONIAE (Pneumococcus)

Characteristics: Large lancet shaped cocci, usually occurring in pairs; sometimes found singly or in short chains. When in pairs, the adjacent ends of the cocci are usually bluntly rounded, the opposite ends are acutely pointed. In films from sputum, blood and cultures on serum containing media, a definite capsule can be seen. Gram-positive; stains well with aniline stains and special capsule stains. Poor growth on plain agar; grows best on blood or serum agar with pH 7.6 to 7.8; colonies on blood agar plate, surface flat and smooth with edge sharply raised from the medium, surrounded by a narrow zone of alpha-haemolysis (green discoloration); some strains (Types III and VIII) give characteristic mucoid colonies. Killed in 20 minutes or less at 55°C. Bile soluble; ferments inulin. 31 distinct serological types have been identified; called D. pneumoniae Type I, II, etc. to XXXIII; however, types 26 and 30 apparently are identical with types 6 and 15, respectively.

Habitat: The principal cause of lobar pneumonia (over 90%); also may cause bronchitis; bronchopneumonia, conjunctivitis, otitis media, brain abscess, meningitis, endocarditis and arthritis. Frequently present in normal mouths. Highly pathogenic for mice and slightly less so for rabbits.

### Identification:

1. Direct microscopy: make spreads of specimen on slide, fix and stain by Gram's method and /or Hiss's capsule stain. Examine for diplococci showing typical morphology; if present confirm by procedures below.
2. Typing by Neufeld Reaction. This is the rapid method of choice for identification of type on materials direct from the patient, giving the type within 30 minutes. It is less applicable to typing of cultures or to detection of type in patients who have received one of the sulphanilamide compounds. Only after pneumococci have been shown by stained spread to be present in appreciable numbers, is this typing effort to be attempted.

(a) Collection of specimen: small sample of sputum, carefully coughed up by the patient from deeper air passages, as free as possible of saliva, is collected in a Petri dish or wide mouthed bottle and should be typed within one hour of collection. Older specimens may be examined only if they have been kept on ice. Preferably the sample is to be collected before beginning treatment with sulfapyridine (which interferes with this test). Samples with few pneumococci, or which otherwise give poor results by this test may be inoculated intraperitoneally into a mouse, and the mouse peritoneal washings 18 hrs. later used for this or other typing effort. Specimens of spinal fluid and cultures in blood or serum broth also may be typed directly.

#### (b) Materials.

- (1) Platinum loop, 1 mm. for transferring sputum.
- (2) Platinum loop, 4 mm. for serum and dye.
- (3) Loeffler's alkaline methylene blue (not required if dye is already in type serum).
- (4) Glass slides and cover glasses.



(5) Typing sera (Rabbit): type I to XXXIII monovalent serum and group mixtures; Mixtures A. (types 1, 2 and 7); B. (3, 4, 5, 6, 8); C. (9, 12, 14, 15, 17); D. (10, 11, 13, 20, 22, 24); E. (16, 18, 19, 21, 28) and F. (23, 25, 27, 29, 31, 32). These sera may be in capillary tubes, each with enough for one test, or in small bulk bottles.

(c) Technique of Test:

(1) Divide 3 clean slides into halves by wax pencil and label halves "A, B, C, D, E, F".

(2) Place tiny flock of sputum in center of an area, with small loop.

(3) Add typing serum, about 5 times as much as specimen used.

(4) Add large loopful of methylene blue. Mix thoroughly.

Apply cover slip.

(5) Let stand for 5 minutes (prepare other slides while waiting)

(6) Examine under oil immersion objective for dark blue diplococci surrounded by unstained area with definite outline. Only a small indistinct capsule can be seen around the pneumococci mixed with heterologous antisera. Large distinct halo surrounds the pneumococci which have been mixed with their type antisera. If none of the mixtures are found positive at first examination, yet pneumococci have been shown to be present by stained spread, reexamine these slides from time to time over a period of 30 minutes.

(7) Positive mixtures having been determined, repeat the test procedure with each serum contained in that mixture, until the positive type or types have been determined.

3. Culture

(a) Streak specimen (sputum, pus, peritoneal washings or mouse's heart blood) on blood plate, incubate 24 hours at 37°C.; then select well isolated typical greenish colonies and examine on stained slide for morphology; transfer suspect colonies to calcium carbonate broth, incubate for 24 hours and examine by confirmatory tests.

4. Animal Inoculation (applicable when other tests are incomplete)

(a) Select small piece of tenacious sputum, wash through three changes of sterile saline, emulsify in mortar with sterile saline.

(b) Inject mouse intraperitoneally with 1 cc. of suspect emulsion or culture. The pneumococci grow rapidly in the peritoneum while most other bacteria die off.

(c) When mouse appears sick, or after 6 to 8 hours, withdraw a drop of peritoneal exudate by a capillary pipette or hypodermic needle punctured through the abdominal wall. Examine microscopically.

(d) If diplococci are numerous, kill the mouse and use peritoneal washings for typing and culturing; also make culture from heart's blood.

(e) If diplococci are not numerous on preliminary trial, retest in 24 hours.

5. Confirmatory tests: when indefinite results are obtained by the above tests, the identify of a pure culture of the suspect organism can be confirmed by the following examinations:



(a) Bile solubility test: add 0.2 cc. of sterile bile to 0.8 cc. of culture; prepare a control by adding 0.2 cc. of sterile saline to 0.8 cc. of culture; incubate 2 hours and note clearing of the turbidity due to solubility in bile of the pneumococcus. Streptococci and other organisms are not bile soluble.

(b) Inulin fermentation: inoculate a tube of inulin -serum water; incubate 37° C. for several days. This medium is usually fermented and coagulated by pneumococci but not by streptococci.

(c) Agglutination tests: macroscopic tests with suspensions of the organisms and anti pneumococcus sera.



NEISSERIA

Characteristics: Gram-negative cocci of variable growth vigor and variable pathogenicity. All members give a positive oxydase reaction.

Habitat: N. gonorrhoeae (gonococcus) is the cause of gonorrhoea; N. intracellularis (meningococcus) is the cause of a specific meningitis; both organisms may be readily demonstrated in the exudates from involved tissues; N. catarrhalis and several other species, which are found in the nose and throat of normal individuals, are sometimes associated with certain epidemics of respiratory or eye infections.

NEISSERIA GONORRHOEAE (gonococcus): A strict parasite of man; found in discharges from the genito-urinary system in acute or chronic gonorrhoea, in the pus from gonorrhoeal conjunctivitis, rarely in the blood stream.

Characteristics: Oval or spherical cocci of moderate size, frequently arranged in pairs with adjacent sides flattened or slightly concave, resembling a pair of kidney beans side by side; in exudates the cocci are fairly regular in size and shape and are usually inside the pus cells; in cultures the cocci show variations in size. They are non-capsulated and Gram-negative. The cocci will not grow on plain agar, enrichment of media is needed; grow on moist chocolate agar at 37°C. in 24 hours to small, round, convex, greyish-white colonies; growth is aerobic, favored by atmosphere of 10% CO<sub>2</sub>. Highly susceptible to inimical agencies: when dried the cocci die in 2 hours; moist heat at 55°C. kills in 5 minutes; quickly killed by 1:4000 silver nitrate; cultures kept at room temperature die in a few days, but at 37°C. they may survive several weeks.

Identification: Microscopic examination only is generally done, cultural confirmation done only under special conditions.

1. Microscopic: Make direct spreads of the infected urethral, cervical or conjunctival discharges on glass slides, fix with heat and stain by Gram's method. Examine the stained preparation for Gram-negative, coffee bean shaped, intracellular or extracellular diplococci having the typical morphology of gonococci. Report whether diplococci are intra or extra cellular, or both. Also report any other bacterial forms present, noting for each whether Gram-negative or Gram-positive and whether coccus or bacillus; also the relative numbers and kinds of tissue cells present.

2. Ordinary culture methods, especially in chronic urethral or cervical infections, will reveal only the secondary organisms which may occur. A special culture program is needed for growing N. gonorrhoeae.

3. Special culture program: The cultural demonstration of the gonococcus is superior to direct spread examinations in cases of chronic gonorrhoea in both sexes and in all cases in the female, especially when material for examination is taken from the cervix.



a. The cultivation of the gonococcus, mixed with freer growing micro-organisms, requires observance of the following special procedures:

- (1) Take specimens of representative material and apply directly to plate media.
- (2) Use a medium such as moist chocolate agar, which will readily grow the gonococcus in mixed culture.
- (3) Grow in 10% CO<sub>2</sub>.
- (4) Identify the gonococcus-meningococcus group by colony form and oxydase reaction.
- (5) Confirm the identification by carbohydrate fermentation tests.

b. Specimen taking and transmission: (Optional methods listed in order of preference.)

- (1) Platinum loop is touched to drop of pus, to urethra or to cleansed cervical os, and is immediately stroked broadly over a warm culture plate at the bedside or clinic chair.
- (2) Sterile swab is similarly contaminated with the suspected material at the bedside or clinic chair, immediately placed in a tube containing 1 cc. of nutrient broth for prompt transmission to laboratory and inoculation of warm culture plate (broad spread of .1 cc. of this broth).
- (3) For delayed inoculation (up to 8 hours), the swab-broth tube #2 is stored in icebox until culture plate inoculation is made.

c. Culture Media: (1) Chocolate agar, soft, moist, warm.

(2) The media of McLeod is elsewhere described.

(3) Difco "Proteose #3 Agar" and "Bacto Hemoglobin" may be combined.

d. Incubation: 37°C. in 10% CO<sub>2</sub> in closed jar, 24 - 48 hours.

e. Examination of Culture: Observation made of two features:

- (1) Colony form: convex, slightly opaque colonies, 1 - 3 mm. in diameter, with undulated margins. Their slight opacity and characteristic undulated margins serve to differentiate them from colonies of streptococci and of diphtheroids.
- (2) Oxydase reaction: Flood a segment of the plate with 1 cc. of 1% aqueous solution of dimethyl paraphenylene diamine hydrochloride (Eastman Kodak Co.). (The McLeod program similarly uses 1% tetramethyl paraphenylene diamine hydrochloride, giving the colonies a bright purple color, is more expensive but has the advantage of a more rapid reaction and not killing the cocci in 30 minutes as does the dimethyl.) Gonococcus colonies develop a pink color which on further oxidation becomes maroon and finally black. Streptococcus and diphtheroid colonies fail to undergo this color change. Caution is indicated not to be misled by a mere darkening of the surrounding media. Spreads made and stained from the oxydase-positive colonies must verify the tinctorial and morphological properties of the micro-organisms as this stain is not entirely specific for the Neisseria group. Medium sized, convex and translucent colonies which give the oxydase reaction may be accepted as gonococci if they consist of Gram-negative diplococci; in cases of doubt, i.e., if appearance of colonies is not entirely characteristic or when the complete identification is of special importance, subcultures are made and the fermentation reactions and ability to grow on ordinary agar are determined. (The dye does not interfere with the staining properties of the gonococcus though it does interfere with its cultivation if it has proceeded beyond the pink stage).

NEISSERIA INTRACELLULARIS

(Meningococcus)

Characteristics: Similar to the gonococcus, but found in different locations and possessed with different invasiveness; distinguishable by serological tests. Divided into five types by serological behavior, types I and II and less commonly types III, IV and V; types I and III, and II and IV, respectively, are very closely related. Responsible for endemic and epidemic cerebrospinal meningitis in man; may be found in and isolated from infected spinal fluid, blood or nasopharyngeal secretions of patients suffering with cerebrospinal meningitis and from the nasopharyngeal secretions of carriers. Highly susceptible to inimical agencies; cocci die in less than 3 hours when dried and kept at room temperature; killed by moist heat at 55°C. in less than five minutes; cultures die in a few days when kept at room temperature.

Identification:

1. Macroscopic appearance of the spinal fluid is to be noted and reported. Normal fluid is water-clear and colorless. Meningitis fluid is more or less turbid. Color, turbidity, blood and clot are to be noted. Blood, if fresh, may have come from the spinal puncture and make examination of the fluid difficult.
2. Microscopic: An immediate presumptive diagnosis of meningococcic meningitis may be made by direct study of cerebrospinal fluid.
  - a. Stained films of suspected spinal fluid: centrifuge the fluid, prepare spreads of the sediment on glass slides, fix and stain by Gram's method. Examine for typical Gram-negative, coffee-bean shaped, intracellular diplococci. If present, they should be considered as meningococci and tentatively reported as such, to be confirmed by culture and agglutination tests. The presence of other organisms and the relative number and kind of tissue cells are also reported.
  - b. Cell counts of spinal fluid: make total and differential counts, comparable to the counting of blood cells. The relative number of polymorphonuclear and mononuclear leucocytes are to be noted - the former are usually enormously increased in cerebrospinal meningitis.
3. Culture of Sediment of Spinal Fluid.
  - a. Plant several loopfulls of sediment on warm blood agar plate.
  - b. Inoculate tube of warm serum dextrose broth with 1 cc.
  - c. Incubate cultures at 37°C. for 18 - 24 hours and observe for the typical Gram-negative, coffee-bean shaped diplococci. Cultures are generally pure; if mixed, pure growth is to be obtained by subcultures on solid media (as for the gonococcus). Pure cultures are used for fermentation tests to rule out N. gonorrhoeae, and for tube-agglutination tests.
4. Culture of blood: This is not a routine procedure; the meningococcus may be recovered from the blood by routine methods, in anomalous infections with septicaemia, with or without meningitis.
5. Culture of nasopharynx: This is done for the detection of carriers only. The nasopharynx of convalescents and of potential carriers are touched with a sterile applicator or inoculating needle, and this inoculum is spread diffusely onto warm blood agar or chocolate agar plates; after incubation at 37°C., suspect colonies are fished to warm serum dextrose broth for confirmation of identify.



6. Agglutination tests of pure cultures: A presumptive slide agglutination may hasten the procedure and cast out atypical organisms. A macroscopic tube-agglutination test with polyvalent meningococcic antiserum is used for final proof of identity. Occasionally type determination will be indicated. Most of the saprophytic Neisseria are salt or serum sensitive; to rule out non-specific clumping it is necessary, in all agglutination tests for meningococci, to run controls using normal horse serum (diluted 1:10) and saline.

a. Presumptive test: Place a drop each of polyvalent antimeningococcic serum (1:10), normal horse serum (1:10) and sterile saline on separate areas of a slide; emulsify bacteria (portion of suspected colony) in each drop; observe for clumping of organisms.

b. Macroscopic test tube agglutination test: Add 0.5 cc. amounts of each sera, diluted to  $\frac{1}{2}$  of titer shown on vial, into labeled tubes; use separate tube for polyvalent and for each type antimeningococci serum (usually I & II only) to be tested; another tube receives 0.5 cc. of normal horse serum (diluted 1:10) and last tube receives 0.5 cc. saline; to each tube add 0.5 cc. of suspension of cocci being tested; incubate overnight at 45 - 55°C. or for 2 hours at 37°C. and overnight in icebox.

c. If the organism is a meningococcus, it should agglutinate in tube containing polyvalent and homologous type serum and not in other tubes. If clumping occurs in either control tube, the test is "unsatisfactory".

7. Fermentation reactions: With material from a pure culture, inoculate tubes of serum water media containing 4 pivotal sugars (see chart) and incubate at 37°C.

### Neisseria catarrhalis

Characteristics: Gram-negative diplococci; in sputum, the organisms are shaped like coffee-beans and may be both intra and extracellular; in cultures, they are generally larger and are found in pairs and tetrads; grow freely, forming large colonies in 24 hours. They are normally found in the nose and throat; have meager pathogenicity; may be found, incidentally, in inflammatory secretions especially of respiratory area. A number of closely related Neisseria, also found in respiratory area, are included on differentiation chart.

#### Identification:

1. Microscopic: Make Gram-stained spreads of the infected secretions and examine for Gram-negative cocci. These organisms are larger than meningococci, may not be arranged in pairs, may be intracellular.

#### 2. Culture:

a. Inoculate plain agar, incubate at 22°C.; N. catarrhalis will grow, gonococcus and meningococcus will not.

b. Pure culture is inoculated into sugar series in serum water media.  
(See chart for results)



## Differentiation of Various Species of Neisseria.

	Dextrose	Maltose	Levulose	Sucrose	Agar growth	22°C. growth	Agg. with Menin- gococci serum	Special colony feature
<u>N. gonorrhoeae</u>	A	-	-	-	-	-	-	Small, round, convex.
<u>N. intracellularis</u>	A	A	-	-	±	-	+	Small, round, bluish-grey.
<u>N. catarrhalis</u>	-	-	-	-	+	+	-	Large, greyish-white.
<u>N. sicca</u>	A	A	A	A	+	+	-	Large, wrinkled, impossible to emulsify.
<u>N. perflava</u>	A	A	A	A	+	+	-	Greenish yellow, adherent to medium.
<u>N. flava</u>	A	A	A	-	-	-	-	Yellow
<u>N. subflava</u>	A	A	-	-	±	±	-	Greenish-yellow, adherent to medium.
<u>N. flavescens</u>	-	-	-	-	?	?	-	Golden-yellow

A indicates formation of acid.

A.M.S. 12/23/40.



## BACTERIOLOGICAL EXAMINATION OF WATER

### References:

1. Standard Methods of Water Analysis. A.P.H.A. 8th Ed. 1936. (for technic)
2. A.R. 40-310, Section IX. (collection and shipment)  
A.R. 40-205 par 10 b (3) - Water supply - laboratory control.

### Collection of Sample:

1. Bacteriological examination of specimens of drinking water from all army stations are made routinely once every month and at more frequent intervals when indicated by local conditions, always supported by sanitary surveys and at times by sanitary chemical examination of the water. These examinations are made locally when laboratory facilities are available, otherwise at the nearest laboratory.

2. Samples, representative of the source, are collected by medical personnel of local station and shipped in Item 18050 (sterile 120 cc. bottle in double mailing container) to corps area or comparable laboratory. The collection must be carefully made to avoid extrinsic contaminating factors, such as would be added by the use of unsterile containers, tap drippings, dead end water, insects and other unrepresentative items.

3. All samples should be identified with the essential information as to exact source, time of collection, special circumstances (if any) and the address of the person to whom the report is to be submitted. The laboratory includes this data in its report and adds the time of beginning examination, and the results of test.

4. Specimens, upon receipt in the laboratory, must be stored in icebox and examined as soon as possible; impure waters should be examined within 6 hours of collection, relatively pure waters within 12 hours. Interpretation of results of examination of waters exceeding this shipment interval must consider the elapsed time and the prospect of bacterial changes in interim.

Equipment required: the apparatus, materials and media requirements are laid down in detail in "standard methods", being in general those for routine bacteriological work with special emphasis on specific details of content of media used. The media for water analysis differs from, and is not interchangeable with, other bacteriological media, for it differs in several features: (a) beef extract, not beef infusion, is always used (b) no sodium chloride is added (c) peptone is contained in reduced amount (.5%) and (d) pH is adjusted to the acid side (6.4 to 7.0).

Required tests: two separate and distinct tests are run on each water sample to determine its potability:

- (1) total bacterial count.
- (2) Completed test for the presence of members of Coli-aerogenes group.



# I. The Total Bacterial Count:

(1) This consists of determination of the colony count given by 1 cc. of water on standard nutrient agar after 24 hours incubation at 37°C.; it is not a true total count for it misses dead bacteria, bacteria that do not grow at 37°C and bacteria that do not form visible colonies within 24 hours under standard conditions.

(2) Only two portions (1. cc. and 0.1 cc.) of the sample are routinely plated; if an exact count of a badly contaminated water is desired, additional plates may be planted with smaller measured amounts of water.

(3) Colony counts of over 200 per cc. for treated waters and 500 per cc. for raw waters (spring, well, etc.) are arbitrarily considered as evidencing sufficient contamination of the water to render it unfit for drinking uses; interpretation of any result must also consider the water source, treatment, and sanitary survey.

## (4) Preparation of plate cultures:

- (a) Two Petri plates are labeled on lid with sample number and amount.
- (b) Sample is mixed thoroughly by shaking vigorously 25 times.
- (c) 1 cc. of water sample is measured, by a sterile pipette, into one plate, .1 cc. into the second plate.
- (d) Nutrient agar is added to each plate: 10 cc. of liquified agar, cooled to 42 - 45°C.
- (e) Mixture of agar and water is effected by tilting and rotating plate.
- (f) Hardening of agar is then permitted by a few minutes rest at room temperature, the dishes inverted and so kept throughout later observations.
- (g) Control plate is prepared by same procedure, less the water sample.
- (h) Incubate all plates at 37°C. for 24 hours (  $\neq$  1 hour).

## (5) Colony Counting:

- (a) Count the number of colonies on the plates, using a lens of 2½ diameters magnification and standard ruled counting plate or using a "Quebec colony counter".
- (b) Calculate colonies per cc. by multiplying the number of colonies on the plate by the fraction of cc. of sample used in the plate which gives the most practical number of colonies for counting, i.e. less than 300 colonies per plate.
- (c) Report colony count, exactly in low counts, approximately in higher counts:

Colony Count of 1 to 50	is reported exactly as counted
" " 51 to 100 "	" to nearest 5.
" " 101 to 250 "	" " " 10.
" " 251 to 500 "	" " " 25.
" " 501 to 1000 "	" " " 50.
" " 1001 to 10,000 "	is " " " 100.

## II. Completed Test for the Presence of Members of the Coli-Aerogenes Group.

- (1) Completed test is indicated when dealing with drinking water examination.
- (2) Partial tests, called in standard methods, the presumptive and confirmed tests, are used for hasty examinations, for raw water in process of purification, for sewage and other known polluted waters where complete test is unnecessary, such as in water purification plants and sewage disposal plants.
- (3) The Coli-aerogenes group includes all aerobic and facultative anaerobic Gram-negative, non-spore-forming bacilli, which ferment lactose with gas formation.
- (4) This group is not pathogenic, not necessarily harmful to the water, but are considered as evidencing faecal pollution and the potential presence of pathogenic faecal organisms of dysentery-typhoid-salmonella or cholera groups which are not so readily detected in routine tests.
- (5) Positive completed tests require the demonstration, in subcultures made from initially inoculated lactose broth cultures, of one or more aerobic plate colonies of Gram-negative, non-spore-forming bacilli which forms gas when again inoculated into a lactose broth fermentation tube.
- (6) Media required:
  - (a) Lactose broth with brom-cresol-purple indicator placed in large test tubes (30 cc.) and small test tubes (10 cc.) each with small inverted test tubes within to demonstrate gas formation.
  - (b) Eosin-methylene blue agar for water ("E.M.B.")
  - (c) Brilliant green lactose bile ("B.G.L.B.") fermentation tubes or an authorized substitute.
  - (d) Nutrient agar slant

### (7) Technique of test:

1st day: (1) Mix water sample thoroughly by shaking vigorously 25 times

- (2) Inoculate 2 small lactose tubes with 0.1 cc. and 1.0 cc. portions, respectively, and 5 large lactose tubes with 10 cc. each of the water sample; label tubes with sample number, and number from 1 to 7.

(3) Place in incubator at 37°C. for 24 hours.

2nd day: (1) Observe lactose fermentation tubes; record presence and percent, or absence of gas formation.



(2) If gas has been formed in any tubes inoculate the following media:

- (a) Streak on E.M.B. Plate: Plant from the tube inoculated with smallest amount of original water sample and showing gas formation.
- (b) Brilliant green lactose bile tubes: plant from at least three (preferably all) tubes showing gas formation, including tubes inoculated with smallest portions of original water sample.

(3) Place original lactose tubes and transplants in incubator at 37°C. for another 24 hours.

3rd day: (1) Make and record 48 hour readings on original lactose tubes; if no gas has been formed in any tube, make negative report; if gas has been formed in tubes inoculated with a smaller portion of water sample than at 24 hours, inoculate E.M.B. plate and B.G.L.B. and proceed as indicated for procedure 2 (second day).

(2) Observe 24 hours E.M.B. plate for typical coli or aerogenes type colonies; if present, select one or more (one of each type present) well isolated colonies and transfer to small lactose fermentation tube and plain agar slant; the presence of typical colonies within 48 hours is recorded in column D, Form 95, M.D. as positive; if no typical colonies are found on plate and gas has been formed in corresponding B.G.L.B. tube, a new E.M.B. plate should be streaked from that tube.

(3) Observe B.G.L.B. tubes for gas formation; gas in any amount is recorded in column C, Form 95, M.D. as positive; reincubate any negative tubes.

(4) Place newly inoculated media and other media as indicated into incubator at 37°C. for 24 hours.

4th day: (1) Observe any 48 hour cultures, (a) E.M.B. plates for typical colonies, or (b) B.G.L.B. tubes for gas formation, and record results; if positive proceed as indicated for procedures (2) and (3) third day; if no typical colonies are present on plate and no gas has formed in any tube of liquid confirmatory media, report as negative.

(2) Observe secondary lactose tubes for gas formation and record results. Reincubate.

(3) If gas has been formed in lactose tube, and not otherwise, make Gram-stained film from corresponding plain agar slant



culture and examine for Gram-negative, non-spore-forming bacilli and record in column F as "C.-A.".

- 5th day: (1) Make 48 hour reading of secondary lactose tube and record.
- (2) If gas formation has occurred in lactose tube that was negative at end of 24 hours, make Gram-stained film and examine as above.
- (3) Prepare report on Form 95, M.D., using a standard remark wherever applicable.

Note: In most infected water samples, gas formation will occur within 24 hours in all cases and the test can be completed as shown in above outline within four days of receipt of specimen; sometimes as indicated in procedure (1) for 3rd and 4th days, lactose fermentation is delayed; in this case proceed as if reaction had occurred within 24 hours but note that five or more days will be required to complete test.

Standard Remarks for Use in Reporting Results of  
Bacteriological Examinations:

- No. 1. Condition: No gas in any lactose tube. Colony count under 200 per cc.  
Remark: Potable bacteriologically. No evidence of fecal contamination.
- No. 2. Condition: No gas in any lactose tubes. Colony count over 200 per cc.  
Remark: Potability questionable. Colony count is high ("very high" if over 1000).
- No. 3. Condition: Gas due to Coli-aerogenes group in one or two large lactose tubes. Colony count low.  
Remark: Potability questionable. Coli-aerogenes group organisms present in one (or two) 10 cc. samples.
- No. 4. Condition: Gas due to Coli-aerogenes group in one or two large lactose tubes. Colony count over 200 per cc.  
Remark: Not potable bacteriologically. Coli-aerogenes group organisms present in one (or two) 10 cc. samples. Colony count high.
- No. 5. Condition: Gas in one or more original lactose tubes. Failure to demonstrate presence of Coli-aerogenes group (E.M.B. plate and B.G.L.B. tubes negative, no gas in secondary lactose tubes, or gas due to spore-forming bacillus). Colony count under 200 per cc.  
Remark: Potable bacteriologically. Gas formation not due to Coli-aerogenes group.
- No. 6. Condition: Same as No. 5, except colony count is over 200 per cc.  
Remark: Not potable bacteriologically. Gas formation not due to Coli-aerogenes group. Colony count is too high.

No. 7. Condition: Gas formation due to Coli-aerogenes in three or more large lactose tubes or in a small lactose tube. Colony count high or low.

Remark: Not potable bacteriologically. Tests reveal evidence of fecal contamination.

No. 8. Special remarks - to be added after use of some other remark.

a. Specimen \_\_\_\_\_ days in transit to laboratory.

b. Evidence of defective packing or collection.

Table No. \_\_\_\_\_. Example of water report, Form 95, M.D., filled out correctly.

Differentiation of Members of the Coli (genus Escherichia)—Aerogenes (genus Aerobacter) group: A satisfactory identification of a Coli-aerogenes group organism as Escherichia coli, E. freundii or Aerobacter aerogenes can be based upon four tests (indol, Methyl red, Voges-Proskauer and sodium citrate). These tests are not routinely run in the Army, but are sometimes requested by a Post Surgeon making a sanitary survey of a water shed. E. coli (Indol +; M.R. +; V.P. - and citrate -) are considered to be of fecal origin; E. freundii (indol +; M.R. +; V.P. - and citrate +) and A. aerogenes (Indol -; M.R. -; V.P. + and citrate +) of non-fecal origin.

The Bacteriological Examination of Swimming Pool Water: The bacteriological examination of swimming pool water is carried out by the technique described for water and the standard of purity recommended is identical with the standard for a potable drinking water. Since most swimming pools contain residual chlorine, sufficient to kill bacteria in the sample between time of collecting and testing, it is required that such samples be collected into sterile water sample bottles containing approximately 0.02 to 0.05 gms. of sodium thiosulphate.



# I. BACTERIOLOGICAL EXAMINATION OF MILK.

## References:

1. Standard Methods for the Examination of Dairy Products, 7th Ed., 1939, published by American Public Health Association.
2. Standard Milk Ordinance and Code of the U.S. Public Health Service.
3. A.R. 40 - 310, Sections XII and XIII.

## Definitions:

1. Raw milk is untreated (except for refrigeration) milk.
2. Pasteurized milk is milk that has been treated with limited heat by one of several methods, in order to kill most pathogenic bacteria.
3. Certified milk is an especially pure raw or pasteurized milk, generally for infant feeding, produced under the supervision of a medical milk commission of the county or state Medical Society, based on requirements of the American Association of Medical Milk Commissions.

a. Certified raw milk: colony count should not exceed 10,000 per cc.

b. Certified pasteurized milk: colony count of not over 10,000 per cc. before, and of not more than 500 per cc. after pasteurization.

4. Standard milk Ordinance and Code classifies and defines milk as:

Grades A, B, C and D Raw.

Grades A, B and C Pasteurized.

5. Grade A Pasteurized Milk is the grade usually sold for drinking purposes; must have colony count of not over 30,000 per cc.; and must be prepared from Grade A (50,000 per cc.), or Grade B (200,000 per cc.) raw milk in plants meeting strict sanitary requirements.

6. Other grades of milk are based on definite sanitary requirements for the production, distribution and bacterial content; the allowable colony counts for raw milks are greater than for the corresponding grade of Pasteurized milk; also, the sanitary requirements are progressively less rigid and the allowable colony counts greater for Grades B, C, and D milk, respectively.

## Collection of Samples:

1. Sample selected should be (a) representative of the lot to be tested, (b) free of extrinsic contamination and so preserved by the use of sterile or contamination-free containers, and (c) iced from the time of collection



to time of laboratory test, to prevent bacterial growth in transit.

2. Bottle should be picked at random from distribution channel, kept on ice and set up in laboratory within 4 hours, if possible.

a. Protect cap and lip of bottle from contamination in transmission by a tight fitting, waterproof covering.

b. Pack in ice in upright position, keeping its temperature under 45°F. until examined.

c. If sample is to be sent to a distant laboratory by mail for direct microscopic count, add 2 drops of formalin for each 10 cc. of milk, fill a sterile 120 cc. glass-stoppered bottle up to the stopper, label "Formalinized", and prepare for mail shipment in a double mailing case.

3. Bulk milk may be sampled at the plant or in distribution by collection with sterile equipment and handled as in #2 above.

4. A plate count at a local laboratory is preferable to a direct count at a distant laboratory.

5. All samples should be properly identified with the essential information as to name of dairy, time of collection, source and grade of milk, preservative used (if any) and the address of the person to whom the report is to be submitted. The laboratory includes this data on its report and adds the time of the start of examination, and results of test.

#### Standard Tests:

The following methods are applicable to samples of milk received under differing conditions and according to the laboratory facilities locally available.

a. Agar plate method: this consists in counting the number of visible colonies of bacteria in a culture made of a measured amount of milk in standard nutrient agar after 48 hours incubation at 37°C. This gives an estimate of the number of living bacteria present in the milk and is the test routinely used in Army laboratories.

b. Direct Microscopic Method: consists of an examination of stained films of milk and cream dried on glass slides. It is used in the central Army laboratories in the making of estimates of the number of individual bacteria (living or dead) in specimens of "formalinized" milk from outlying stations where laboratory facilities for agar plate method are not locally available. See "Standard Methods for the Examination of Dairy Products" for technique.

c. Methylene Blue Reduction Method: usually known as the "Reductase test", is based on the fact that color imparted to milk by a small amount of methylene blue will disappear more or less quickly from incubated milk as

a result of the consumption of the dissolved oxygen by growing bacteria. The results of this test compare favorably with those obtained by other methods. Due to the small amount of equipment, space and experience required, it is applicable to small laboratories and in isolated posts.

d. Sediment test: this depends upon appearance of standard filter discs after passage of 1 pint of milk. It is used as an index to the cleanliness of milk. It has little utility in Army laboratories.

e. Tests for Specific Types or Groups of Bacteria, such as, coliaerogenes group, hemolytic streptococci, tubercle bacilli and Brucella are described in Standard Methods. These tests are similar to the routine laboratory examinations for those micro-organisms and are seldom done routinely.

#### Standard Plate Count:

1st day. 1. Agitate milk sample thoroughly, preferably by pouring back and forth from one sterile container to another.

2. Using sterile dilution bottles containing exactly 9.0 cc. or 99 cc. of distilled water, prepare a series of dilutions depending upon expected colony count, (based on Grade of milk and results of previous examinations); the plate to be counted should have between 30 and 300 colonies; routinely dilutions of 1/100, 1/1000 and 1/10,000 are prepared.

3. Mix each dilution as prepared, by shaking rapidly up and down 25 times in arc of 1 foot. Transfer 1.0 cc. of each dilution to properly labelled Petri dish.

4. Add tube of standard nutrient agar (tryptone-glucose-extract-milk agar), previously melted and cooled to 42°C., to each Petri dish and mix with sample by rotating and tilting the dish carefully. Allow to cool.

5. Incubate at 37°C. for 48 hr. (plus or minus 3 hrs.)

3rd day. 1. Select the plate containing between 30 and 300 colonies and count all colonies including those of pin-point size. The use of a Quebec colony counter is recommended. If only a fraction of the plate is counted, determine total plate count by multiplying the average number of colonies per sq. cm. by a variable factor depending upon the average inside diameter of the Petri dishes being used (90 mm. multiply by 63.5; 91 mm. by 65; 92 mm. by 66.5).

2. Multiply the number of colonies found by the dilution factor to find colony count per cc. of sample.

3. Report: "Standard Plate count, \_\_\_\_\_ per cc."

Methylene Blue Reduction Method: This test is most applicable to raw milk; aseptically drawn normal milk from healthy udders seldom, if ever, reduces methylene blue in less than 10 hours.



a. Methylene Blue Reagent. Use only certified methylene blue thiocyanate tablets. Prepare fresh reagent weekly by dissolving one tablet of dye in exactly 200 cc. (at room temperature) of sterile or freshly boiled distilled water.

b. Technique of Test.

1. Mix sample thoroughly and transfer 10 cc. to a 12 to 15 by 150 mm. test tube, fitted with a rubber stopper.

2. Add 1.0 cc. of the methylene blue reagent.

3. Stopper the tubes immediately, label indelibly and place in water bath at 37°C. Invert the tube once at end of five minutes, after which avoid agitation that might disturb the cream layer.

4. Observe tubes at frequent intervals (15-30 minutes) and record the end point (disappearance of the blue color from at least four-fifths of the contents of the tube).

c. Interpretation of Results:

Class 1. Excellent, not decolorized in 8 hours.

Class 2. Good, decolorized in less than 8 hours but not less than 6 hours.

Class 3. Fair, decolorized in less than six hours but not less than 2 hours.

Class 4. Poor, decolorized in less than 2 hours.

Tests for presence of coli-aerogenes group, consists of inoculating five tubes each of various portions (10 cc., 1.0 cc., 1/10 cc., 1/100 cc., etc.) of the milk to be tested into Brilliant Green Lactose Bile, or Formate Ricinoleate broth. If gas is formed, continue as with water for definite identification.

## II. BACTERIOLOGICAL EXAMINATION OF CREAM.

Estimations of the bacteriological content of cream samples are made by using same methods as those for milk with these exceptions:

a. Measuring sample: Mix sample; weigh 1.0 gm. aseptically into a sterile butter-boat or directly into a dilution bottle;

b. Dilutions used: The allowable bacterial content of cream (50,000 to 100,000 colonies per cc.) is greater than for milk; carry dilutions one or two steps farther when making agar plate count.

## III. DETERMINATION OF THE NUMBER OF BACTERIA IN PLAIN ICE CREAM:

In the bacteriological examination of ice cream and of ice cream mix before



it is frozen, follow the same general methods as for milk; using (1) the agar plate method and (2) direct microscopic count and (3) sometimes testing for coliform group. Collect samples of at least 50 cc. amounts in unopened cartons or in sterile wide-mouth, 125 cc. bottles, fitted with ground glass stoppers or metal caps. In sampling bulk ice cream, remove top inch of cream with sterile spoon, discard this and use a second sterile spoon to collect sample; collect representative samples of ice cream mix at periodic intervals. Send to laboratory immediately for examination; if laboratory is at some distance, keep sample properly refrigerated by packing in dry ice or in water containing cracked ice.

#### Standard Plate Method:

a. Preparation of sample: Melt the frozen ice cream by placing the container in a water bath at  $45^{\circ}\text{C}$ . for a period not to exceed 15 minutes.

#### b. Method of making dilutions:

1. Volumetric Method: To reduce the percentage of error when using materials of high viscosity (melted ice cream, ice cream mix, condensed milk, etc.) it is necessary to use large amounts in making the first dilution. To make 1:10 dilution, use 11 cc. of sample to 99 cc. of sterile water in dilution bottle. Most applicable to plant control work.

2. The Gravimetric Method is more accurate and should be used for most Army laboratory examination.

(1) Bring the sample to a suitable degree of consistency (about  $10^{\circ}\text{C}$ .) by allowing to stand at room temperature or by heating in water bath at  $43 - 46^{\circ}\text{C}$ .; using either method the total time required must not exceed 15 minutes.

(2) Obtain (a) a butter-boat or similar piece of apparatus, sterilized in cotton-plugged test tube and (b) a standard dilution bottle containing 99 cc. of sterile distilled water, and having opening of such size as readily to take the butter-boat.

(3) Remove the cotton plug from the test tube and slide the butter boat forward until it projects about  $3/4$  inch beyond end of test tube. Do not allow boat to touch any contaminated object. Weigh test tube and butter-boat to the second decimal place.

(4) Pipette one gram, or slightly more, of the sample into the butter-boat and again weigh to the second decimal place.

(5) Allow the butter-boat and contents to slide into the dilution bottle.

c. Technique of test: make further dilutions, if required, and continue as for examination of milk.



### Preparation of Autogenous Vaccines.

Selection of Culture: An autogenous vaccine is one prepared from a culture isolated directly from the patient who is to be treated with that vaccine. With the infected materials, such as pus or tissues, prepare Gram-stained films and examine them for type organisms present. Inoculate blood agar and infusion agar plates and tube of infusion broth; incubate for 24 to 48 hours at 37°C. Examine the plates for predominating types of colonies; make Gram-stained preparations from each type. Also examine stained preparations from the broth culture for presence of any organism not found on plate cultures and inoculate upon plate media if indicated. If a single type organism is found in pure culture, transplant to the desired media for the vaccine. When mixed cultures are obtained, select isolated colonies of the type desired and transfer to plates to isolate pure cultures; incubate for growth; and again check for purity of cultures. If several organisms are present, presumptive evidence as to which organism is concerned in the infection can sometimes be gained (1) by agglutination of the organism by patient's serum or (2) by positive skin reaction on intradermal injection of the vaccine. Do not use spore-forming bacilli or any organisms which are obviously contaminants. Staphylococci and Streptococci are the organisms most frequently used for production of autogenous vaccines.

Preparation of Suspensions: a. Agar slant cultures: Most bacteria are best grown by transferring to three or four infusion agar slants for 24 to 48 hours; blood or serum agar are required for growing other organisms. Add 2 or 3 cc. of sterile saline to each tube and emulsify bacteria by shaking or by agitation with a platinum loop. The suspension should be quite heavy. If any clumps are observable in the bacterial suspension, transfer to a sterile flask or bottle containing beads and shake thoroughly; then filter aseptically through several layers of gauze, held in a small funnel, to break up any remaining clumps or to remove any particles of culture media.

b. Broth cultures: If broth cultures are used, sediment the bacteria by centrifuging and resuspend in sterile saline; centrifuge a second time and resuspend in sufficient saline to give a heavy suspension. (Note: it is sometimes desirable to prepare a vaccine containing both bacteria and their exogenous toxins; in this case, do not centrifuge or wash in saline.) Break up clumps of bacteria and continue as above.

Killing of Bacteria: Place the bacterial suspension in a sterile ampule (Standard item no. 40074) and seal hermetically. Weigh the ampule with sheet lead and immerse in a water bath at 56° to 60°C. for one hour. Remove from the water bath and culture for sterility by inoculating 0.25 cc. portions into suitable media and incubating aerobically and anaerobically for 2 or 3 days.

Determining Bacterial content: a. Wright's method: Prepare a capillary pipette with long capillary section; mark about 1/2 inch from tip. Draw up bacterial suspension to the mark, then a small air bubble, and then blood from the finger tip to the same mark. Mix quickly on a slide; make thin film and



stain by Wright's method. Count the number of red blood cells and the number of bacteria in several areas. Use the following formula to determine the number of bacteria per cc. of suspension:

$$\frac{\text{Bacteria counted}}{\text{Red cells counted}} \times 1000 \times 5000000 = \text{No. of bacteria per cc.}$$

b. Nephelometric method (McFarland): This is the preferred method, when the suspension contains no coloring matter. It consists in comparing the opacity of the bacterial suspension with that of various densities of barium sulfate in a series of test tubes.

(1) Preparation of standards: Prepare 1% aqueous solutions of C.P. sulfuric acid and C.P. barium chloride. To a series of 10 Pyrex glass test tubes of uniform size add increasing amounts of the BaCl solution, starting with 0.1 cc. in first tube, increasing the quantity by 0.1 cc. in each succeeding tube, to add 1.0 cc. in tenth tube. Then add to each tube enough H<sub>2</sub>SO<sub>4</sub> solution (9.9 cc. to 9.0 cc., respectively) to bring the total volume to 10 cc. Seal hermetically and label serially from 1 to 10.

The density of the suspensions in these tubes corresponds approximately to from 300 million organisms per cc. for first tube to 3000 million per cc. for tenth tube, increasing by 300 million bacteria for each succeeding tube from 1 to 10.

(2) Technique: Place a measured quantity (1.0 cc. or more, depending on density) of the bacterial suspension in a test tube of the same diameter and color as those used for the standard. Dilute by adding a measured amount of sterile saline to the density of one of the standards; shake well during process.

(3) Calculation of bacteria per cc.: The approximate number of bacteria per cc. of suspension corresponds to the tube matched, times the dilution. For example, if 1.0 cc. of suspension was diluted to 4 cc. to match tube no. 4, it contains 1,200,000,000 x 4, or 4,800,000,000 organisms per cc.

Note: A bacterial standard containing 1000 million organisms per cc. can be prepared by adding 4 cc. of sterile saline to 8 cc. of triple typhoid vaccine (1500 million bacteria per cc.) and sealing in Pyrex glass test tube.

Preparation of Vaccine: Most autogenous vaccines for treatment are prepared in a concentration of 1 billion organisms per cc. To prepare a definite quantity, say 30 cc., of vaccine of this strength from the bacterial suspension above, find the number of cc. of suspension required by use of this formula:

$$\frac{30 \text{ (no. of cc. of vaccine required)} \times 1000000000 \text{ (desired strength)}}{4,800,000,000 \text{ (strength of suspension)}} = 6.25 \text{ cc.}$$

After standardization, add phenol to a final concentration of 0.5% (1 cc. of stock 5% phenol to 9 cc. of vaccine) for preservation. Dispense in a properly labelled, sterile vaccine bottle, closed with a rubber stopper.

GRAM-NEGATIVE, AEROBIC, NON-SPORE-FORMING ENTERIC BACILLI.  
(FAMILY ENTEROBACTERIACEAE)

Characteristics: Gram-negative rods, widely distributed in nature. Grow aerobically. Many species are parasitic for man, several of which cause typical disease; other species are saprophytes, or parasites on plants and animals. Grow well on ordinary culture media. All species attack certain carbohydrates forming acid, or acid and visible gas. May be motile or non-motile. Non-spore forming. Has been divided into five tribes, only three of which (Eschericheae, Proteae and Salmonelleae) contain species of interest in Medical Bacteriology. All of these bacteria are morphologically similar. They have many other characteristics in common, and serological as well as cultural methods may be required to definitely identify a member of the group.

THE COLI-AEROGENES GROUP.  
(Tribe Eschericheae)

Characteristics: Motile or non-motile rods, commonly occurring in the intestinal canal of normal animals, in the respiratory tract of man, or widely distributed in nature. All ferment dextrose and lactose with the formation of acid and visible gas. Do not liquefy gelatin except slowly by one species (Aerobacter cloacae). Separated into three genera on basis of results of methyl red test, Voges-Proskauer test, and ability to utilize citric acid as sole source of carbon. See table below.

Genus and species	Methyl red test.	Voges- Proskauer test.	Indol test.	Citrate utili- zation.	Gelatin lique- faction.	H <sub>2</sub> S formed.
<u>Escherichia coli</u>	+	+	+	-	-	-
<u>E. freundii</u>	+	-	(+)	+	-	+
<u>Aerobacter aerogenes</u>	-	+	(-)	+	-	(-)
<u>A. cloacae</u>	-	+	-	+	+	(-)
<u>Klebsiella pneumoniae</u>	(+)	(-)	-	(+)	-	-

Note: Some species give variable results; (+) or (-) indicates usual reaction.

Escherichia Coli.

Characteristics: Coccoid to long rods, occurring singly, in pairs and long chains. Gram-negative. Motile or non-motile. Not usually capsulated. Ferments many carbohydrates, including dextrose and lactose, with formation of acid and gas. The large number of species formerly identified on basis of motility and carbohydrate fermentation are now included within this species as varieties.



Habitat: Occurs in normal intestinal tract of animals; frequently found in soil and water, as a result of fecal contamination. Sometimes acquires pathogenic power and may cause local or general infections; frequently causes infections of the genito-urinary tract; invades the circulation in agonal stages of diseases.

Identification: 1. For isolating E. coli from water and sewage see section on "Bacteriological Examination of Water".

2. For E. coli in feces, urine, etc., follow the procedure outlined under examination of feces for E. typhosa and identify according to the reactions in chart above.

#### Aerobacter aerogenes.

Characteristics: Short rods with rounded ends, usually shorter and plumper than E. coli. They are aerobic, Gram-negative, non-spore-forming and frequently capsulated. Ferment many carbohydrates, including dextrose, lactose and glycerol, with formation of acid and gas. Do not liquefy gelatin. Colonies on solid media are large and very viscid.

Habitat: Widely distributed in nature; normally found on grains and plants, sometimes found in the intestinal canal of man and animals. It has been reported as the cause of cystitis.

Identification: 1. See section on "Bacteriological Examination of Water" for method of isolating A. aerogenes from water.

2. Isolate organism from feces, food, and soil by plating on eosin methylene blue agar or other media as described under E. typhosa and identify by characteristic biochemical reactions shown in above table.

#### Klebsiella pneumoniae.

Characteristics: Short, plump, non-motile, Gram-negative rods; aerobic, growing well on ordinary media; produces a large, mucoid colony on solid media. It has a large capsule which can be demonstrated readily in spreads from sputum, animal exudates and other pathological material. Ferments dextrose, levulose, galactose, saccharose and usually lactose with production of acid and gas.

Habitat: Common commensal in respiratory tract; occasionally found in soil, dust and water. Associated with pneumonia and other inflammations of the respiratory tract. Occasionally found in various suppurative lesions of the body, and may give rise to septicemia.

Identification: 1. Examine stained spreads from pus, sputum, or fluid from lesions for Gram-negative encapsulated bacilli.

2. Inoculate eosin methylene blue agar plates or other media. Examine for mucoid colonies consisting of bacilli with typical morphology. Identify suspected colonies through cultural and biochemical tests.

3. Blood culture may be made by usual methods; identify any suspect colonies as above.



### Genus Proteus.

Characteristics: (The only genus in tribe Proteae.) Consists of highly pleomorphic, Gram-negative rods; filamentous and curved rods, and involution forms are common. Generally actively motile. Characteristically produce ameboid colonies on moist media and decompose proteins; gelatin is rapidly liquefied by most species. Ferment dextrose and generally sucrose, but not lactose, with formation of acid and small amount of gas. Usually Voges-Proskauer test is negative. Genus consists of 8 species; type species is Proteus vulgaris.

Habitat: Putrefying animal and vegetable materials; found in feces, soil and gunshot wounds. Certain Proteus strains, identified as X19, X2, and X Kingsbury, originally isolated from typhus fever cases, are used as antigens in the Weil-Felix test (see section on Rickettsiae). One species, P. morgani, has been reported as the cause of mild enteritis.

Identification: 1. Most laboratories roughly identify any Gram-negative, motile bacillus that produces an ameboid colony on moist agar at 37°C. as belonging to the Proteus group and do not classify further.

2. However, one species of the genus, Proteus morgani, produces the typical ameboid colony only when grown on 1% agar at 21-28°C. Isolate pure cultures of this organism as described under E. typhosa, and identify on basis of fermentation of dextrose and other hexoses only, with formation of acid and slight amount of gas.

### TYPHOID-DYSENTERY AND PARATYPHOID-ENTERITIS GROUPS. (TRIBE SALMONELLEAE)

Characteristics: Motile or non-motile, Gram-negative rods; grow aerobically; non-spore-forming; Voges-Proskauer test negative; gelatin not liquefied; and no spreading growth. Attack many carbohydrates with formation of acid, or acid and gas. Certain species of genus Shigella and genus Elberthella attack lactose with gas formation only. Tribe consists of three genera; genus Salmonella organisms ferment dextrose with the formation of acid and usually gas; genus Eberthella and genus Shigella organisms ferment dextrose with formation of acid, but no gas, Elberthella being motile and Shigella non-motile.

### GENUS SALMONELLA.

The organisms of this genus are defined as: usually motile, but non-motile forms occur. Attack numerous carbohydrates with the formation of acid, and usually gas; lactose, saccharose and salicin are never attacked. Do not form indol or liquefy gelatin. Differ from coli-aerogenes group in failing to ferment lactose; and from typhoid-dysentery group in forming gas from dextrose. Can be separated into 37 species, several of which are pathogenic for man, causing a typhoid-like fever, food poisoning, or an acute gastro-enteritis. All species pathogenic for man are motile.

Important Species: 1. S. paratyphi, the cause of paratyphoid A fever in man. Characteristic reactions: never ferments xylose, rarely able to produce H<sub>2</sub>S, and fails to utilize citrate and d-tartrate.

2. S. schottmuelleri, the cause of paratyphoid B fever in man.

Characteristic reactions: ferments xylose and usually attacks inositol; H<sub>2</sub>S formed; citrate +; and tartrate usually -.

3. S. typhimurium, a natural pathogen of rodents, especially mice, and many other animals; causes food poisoning in man.

Characteristic reactions: very difficult to distinguish from S. schottmuelleri, by means of either biochemical or serological reactions; most reliable tests for separating them being, (a) S. typhimurium is usually tartrate  $\neq$ , and (b) agglutination reactions with "H" antigens of organisms in the specific phase.

4. S. enteritidis, and its varieties, are widely distributed among animals; sometimes the cause of food poisoning in man.

Characteristic reactions: ferments xylose, but never attacks inositol;  $H_2S$   $\neq$ ; citrate  $\neq$ ; and tartrate  $\neq$ .

5. S. hirschfeldii, the cause of a typhoid-like fever in man, sometimes referred to as paratyphoid C. bacillus; found principally in Europe.

Characteristic reactions: biochemical reactions similar to those of S. enteritidis; serologically, closely related to S. choleraesuis.

6. S. choleraesuis, two varieties, causing American and European hog cholera, respectively; occasionally infect man.

Characteristic reaction: fails to ferment arabinose, a carbohydrate attacked by other Salmonella.

Identification: 1. Isolate paratyphoid fever group from feces, urine or blood as described under E. typhosa.

2. Food poisoning group. Isolate pure cultures from feces or food (see section on Food Poisoning).

3. Identify pure cultures by means of carbohydrate fermentations and other biochemical tests (see section on Classification of Bacteria) and by agglutination reactions.

4. The Salmonella group, including E. typhosa, is very complex, serologically. Each species possesses from one to three distinct antigenic components in the body of the bacillus ("O" antigens) and other distinct components in the flagella ("H" antigens), the latter occurring in many species in two alternate phases, the specific phase and the group phase, each possessing different antigens. The same antigenic components may be found in several different species, in various combinations. However, most strains of the pathogenic species listed above can be definitely classified on basis of (a) source of specimen, (b) biochemical reactions and (c) series of agglutination tests.

#### GENUS EBERTHELLA.

The organisms of this genus are defined as Gram-negative, motile rods, generally occurring in the intestinal canal of man, usually in different forms of enteric inflammation. Attack dextrose and several other carbohydrates with the formation of acid, but no gas; certain non-pathogenic species may attack lactose, saccharose and/or salicin with formation of acid, but no gas. E. typhosa is the only species regularly pathogenic for man.



Eberthella typhosa.

Characteristics: Actively motile, Gram-negative rods, possessing the general features of the tribe and genus. Never attack lactose, saccharose or salicin. The normal smooth motile form has one somatic and one flagellar antigen, thus producing both H and O agglutinins; non-motile variants are rare; the somatic antigens are related to those of Salmonella enteritidis and a number of other species of Salmonella. Colonies on plain agar, after 24 hours incubation at 37°C., are smooth, round, domed, grayish in color, transparent to opaque, with entire edge; after cultivation on artificial media, rough type variants may develop. See table in section on "Classification of Bacteria" for biochemical characteristics and table in this section for reaction on Russell's double sugar tubes, and for type colonies on differential plate media.

Habitat: Found in feces and blood, and occasionally in bile and urine, of patients ill with typhoid fever of which it is the causative agent; also present in feces, urine and bile of carriers.

Laboratory Examination of Specimens.

The Specimens to be examined will usually consist of blood, feces, urine or bile of suspected cases of typhoid or paratyphoid fever and of bile, feces and urine of carriers for cultural study; also, of serum from patients for agglutination (Widal) test.

1. Microscopic examination.— This is of no value.

2. Culture.— a. Feces, urine, bile, etc.:

(1) Spread the material, suspended in broth or saline if solid feces, over the dry surface of eosin-methylene blue agar, Leifson's desoxycholate-citrate or other special differential media in Petri dishes, in such a manner as to insure the growth of well isolated colonies. Also, inoculate specimen into tube of selenite broth or bile broth.

(2) Incubate 18-24 hours at 37°C.

(3) Study the plate cultures carefully, select several well isolated colonies of the type desired (see table) and from each inoculate Russell's double sugar (RDS) tube and plain agar slant.

(4) After 24 hours incubation examine cultures for type reaction on R.D.S., motility and Gram-staining properties.

(5) Identify any suspected pure culture (a) by inoculating various carbohydrate media and media for the other biochemical tests, and (b) by setting up macroscopic agglutination tests against known type antisera (E. typhosa, S. paratyphi, and S. schottmuelleri; other antisera may be used, if indicated).

(6) If at end of 24 hours plate cultures show no colonies of the type produced by pathogenic organisms, streak new set of plates from the broth culture and reincubate old plates for an additional 24 hours before discarding as negative.



b. Blood: Blood for culturing should be taken early in the disease, preferably during first week.

(1) Obtain 10-15 cc. of citrated or defibrinated blood; whole blood can be used for immediate inoculation of media at bedside.

(2) Inoculate (a) flask containing 100 cc. of bile broth, 1% dextrose infusion broth or brilliant green broth with 2 to 5 cc. of blood, (b) two agar pour plates with 1.0 cc. of blood each and (c) streak 2 or 3 loopfuls of blood on eosin methylene blue agar plate.

(3) Incubate at 37°C. and make daily transfers to blood agar and E.M.B. agar plates. If colonies develop, transfer to Russell's double sugar and identify by the procedure outlined above.

3. Serological examination.—a. A macroscopic tube-agglutination test, as indicated above, should be used to confirm the identity of an organism isolated from cultures; use suspension of suspected culture as antigen along with known type antisera.

b. Widal test: After the first or second week, demonstrable antibodies, including agglutinins, develop in the blood of patients with an enteric fever. These may be demonstrated by Widal test. This test consists of a macroscopic (preferred) or microscopic agglutination test, using the patient's serum, and stock E. typhosa "H", E. typhosa "O", S. paratyphi and S. schottmuelleri antigens.

#### Genus Shigella.

Characteristics: Small, Gram-negative, non-motile rods. Attack a number of carbohydrates with formation of acid but no gas.

Habitat: Several species are pathogenic for man, causing bacillary dysentery; other species may be found in the normal human intestinal tract; several species are pathogens of fowls and other small animals.

#### The Dysentery Group.

1. Shigella dysenteriae (Shiga): A cause of dysentery in man and monkeys. Produces acid but no gas from dextrose, levulose and a few other carbohydrates. Never attacks mannitol, maltose, lactose or sucrose. Indol not formed. Serologically homogenous and different from the other species of Shigella.

2. Shigella sp. (Newcastle type): A cause of human dysentery. In peptone water solution, dextrose, maltose and occasionally dulcitol are fermented with acid production; lactose, mannitol and saccharose usually not fermented. Peculiarities of the organism are (1) occasionally a slight bubble of gas is produced from dextrose and dulcitol, (2) when dissolved in beef extract broth, dextrose, dulcitol and maltose are always fermented to acid and gas. Indol not formed. Serologically homogenous and not agglutinated by antisera prepared against S. dysenteriae or S. paradysenteriae.

3. Shigella paradysenteriae: A cause of dysentery in man, and of summer diarrhoea in children. Produces acid but no gas from dextrose and mannitol; some strains attack maltose or saccharose; dulcitol and lactose never fermented.

Indol formation is variable. Has been divided into five races ("V", "W", "X", "Y" and "Z") by agglutination tests based upon the preponderance of one or another of four antigenic components, V, W, X and Z; considerable cross agglutination between races; serologically distinct from S. dysenteriae and Newcastle's bacillus; slight cross agglutination with S. sonnei, S. alkaescens and S. madampensis.

4. Shigella alkaescens: Isolated from human feces and intestines; pathogenicity doubtful. Ferments dextrose, mannitol, maltose, dulcitol and sometimes saccharose; never attacks lactose. The most characteristic reaction is an initial and lasting, intense alkalinity produced in litmus milk. Serologically homogenous and distinct except minor cross agglutination with S. sonnei, S. paradysenteriae and S. madampensis.

5. Shigella sonnei: A cause of mild dysentery in man, or of summer diarrhoea in children. Ferments dextrose, mannitol, maltose, lactose, saccharose and several other carbohydrates with formation of acid, but no gas; dulcitol is never, and xylose seldom, attacked; fermentation of substances other than the monosaccharides may require days or weeks. Indol not formed. Serologically divisible into two types; some cross agglutination with S. paradysenteriae, S. alkaescens and S. madampensis.

6. Shigella madampensis (S. dispar): Isolated from human feces; apparently not pathogenic. Fermentation reactions similar to those of S. sonnei. Indol is formed. Antigenically heterogeneous; may show slight cross agglutination with S. paradysenteriae, S. alkaescens and S. sonnei.

#### Laboratory Examination of Specimens.

1. Microscopic examination: In bacillary dysentery, especially in infections with S. dysenteriae (Shiga), an early presumptive diagnosis can usually be made by direct microscopic examination of fresh fecal discharges.

a. Select portions of a very fresh specimen containing bits of mucus, bloody feces or shreds of the exudate. Prepare (1) thin films on slide and (2) cover slip preparations, both unstained and stained with Loeffler's methylene blue or 1% aqueous solution of brilliant cresyl blue, in order to study the cells present.

b. If the disease is the bacillary type of dysentery, microscopic examination will show blood in varying amounts, but usually abundant early in the disease; polymorphonuclear neutrophils form about 90% of the exudate, and many of these show nuclear degeneration (ringing), while the cytoplasm frequently contains fat; endothelial macrophages, which are present in varying numbers, are actively phagocytic and frequently contain engulfed bacteria, erythrocytes and leukocytes; these undergo degeneration and form "ghost cells"; plasma cells are present and are more abundant early in the disease; bacterial content is scanty.

c. For characteristic findings in amoebic dysentery stools, see section on Protozoology.

2. Cultural examination: Shigella may be isolated from the feces of patients and carriers by the methods indicated under Eberthella. However,



both eosin methylene blue agar plates and desoxycholate-citrate agar plates should be inoculated, routinely, since the latter is an especially favorable culture medium for Shigella.

3. Serological examination: a. The suspected organisms may be identified by agglutination tests using polyvalent and species specific antisera; S. dysenteriae, S. paradysenteriae, S. sonnei and polyvalent (Shiga, Sonne and paradysentery) antidysenteric antisera are generally used.

b. Agglutination tests, using serum from patient against known antigens, are of limited value.

Characteristic Reactions of Gram-negative Intestinal Bacilli on Russell's Double Sugar Medium.- Phenol red Indicator (Alkaline is red, Acid is yellowish).

	Slant	Butt
Mechanism	The small amount of acid produced by dextrose (0.1%) is diffused, leaving alkaline slant. The larger amount of acid from lactose (1%) gives acid slant.	Organisms producing acid from either dextrose or lactose give acid butt. <u>Salmonella</u> give acid and gas (bubbles in medium). Typhoid-dysentery group produce acid only from dextrose
Genus <u>Escherichia</u>	Acid	Acid and gas (///).
Genus <u>Aerobacter</u> and Genus <u>Klebsiella</u>	Acid, returning to neutral or alkaline after several days.	Acid and gas (///).
Genus <u>Salmonella</u>	Alkaline	Acid and gas (//).
<u>Shigella dysenteriae</u> and <u>Shigella paradysenteriae</u>	Alkaline	Acid.
<u>Shigella sonnei</u> and <u>Shigella madam-pensis</u>	Alkaline. Small acid producing daughter colonies may be formed after several days.	Acid.
<u>Eberthella typhosa</u>	Alkaline	Acid.
Genus <u>Proteus</u>	Alkaline	Acid and gas (/).



Colony Characteristics of Gram-negative Intestinal Bacilli  
on Differential Plate Media.

	:	: a. Desoxycholate agar.
Medium	: ; Eosin methylene blue agar	
	:	: b. Desoxycholate-citrate agar
	: ; Coli-aerogenes group	: Non-lactose -fermenting
	: ferment lactose and grow	: organisms grow into small,
	: into large, opaque colon-	: clear, colorless, translucent
	: ies; also absorb dye to	: colonies.
Mechanism	: give color to colony	: On a lactose fermenting
	: ; The non-lactose-fermen-	: organisms produce large, reddish
	: ting pathogenic species	: colonies. Growth on b is same
	: develop as small, color-	: except greater inhibition of
	: less, translucent colonies	: coli-aerogenes group.
	:	: a. Large opaque, reddish
	: ; Large colonies with	: colonies; occasionally have
	: large, dark, almost black	: colorless rim.
Genus <u>Escherichia</u>	: centers, and with greenish	: b. Much inhibited; if present,
	: metallic sheen.	: growth is pink and opaque,
	:	: opacity spreading to surroun-
	:	: ding medium.
	: ; Large pinkish mucoid	: a. Similar to <u>Escherichia</u>
Genus <u>Acrobacter</u>	: colonies with small,	: colony except larger and
and	: dark brown or black	: mucoid.
Genus <u>Klebsiella</u>	: centers; rarely show	: b. Much inhibited; same as
	: metallic sheen.	: <u>Escherichia</u> shown in b above.
	:	:
	: ; Translucent, colorless	: Large translucent colonies,
	: or pinkish colonies,	: domed, shiny, smooth and
Genus <u>Salmonella</u>	: usually slightly larger	: colorless.
	: than <u>E. typhosa</u> ;	:
	: later have bluish tint.	:
	:	:
	: ; Small translucent,	:
<u>Shigella dysenteriae</u>	: colorless colonies	: Same as <u>Salmonella</u> colonies
and <u>Shigella</u>	:	: except smaller.
<u>paradysenteriae</u>	:	:
	:	:
	:	: Same as <u>S. dysenteriae</u> colonies
<u>Shigella sonnei</u> and	: Small, translucent,	: during first 24 hours, later
<u>S. madagascariensis</u>	: colorless colonies. Later	: may show reddish daughter
	: may ferment lactose.	: colonies, or entire colony
	:	: may become red.
	:	:
	: ; Translucent colonies;	:
	: Translucent, colorless	: domed, shiny, smooth and
<u>Eberthella typhosa</u>	: colonies.	: colorless.
	:	:
	: ; Translucent, colorless	:
<u>Alcaligenes</u>	: colonies.	: Similar to <u>E. typhosa</u> .
<u>faccalis</u>	:	:
	:	:
	: ; Translucent colorless	: Same as <u>Salmonella</u> ;
Genus <u>Proteus</u>	: colonies; slight	: spreading inhibited.
	: spreading.	:
	:	:



Cl.parabotulinum(Types A & B)and Cl.botulinum(Type C)

Habitat: These are primarily saprophytes of the soil, may occasionally be found in the intestinal tract of domesticated animals and on various foods contaminated by soil or dust. They are not infective to man or animals but do produce disease by means of the violent poison, toxin, it may produce in foods which act as culture media for its saprophytic growth. This toxin is not formed within the body. This poisonous toxin, variously applied, produces "botulism" or food poisoning in man, "forage poisoning" in animals or "limber neck" in poultry. The living organism may be sought for in the infected food but not in the poisoned man or animal for it is not an infection. Botulism may be associated with meat or meat products, fruits, vegetables, canned goods and various pickled and preserved foodstuffs. Broth cultures injected subcutaneously in mice, g.pigs, rabbits, cats, monkeys prove fatal in 1 to 4 days.

Morphology and Staining: Large sporulating rods with parallel sides and rounded ends, occurring singly or in chains. Slightly motile; not-capsulated. Gram-positive. Spores are oval, larger than the bacilli and usually situated at or near the end. Spores form best in sugar free media at a temperature of 20 to 25°.

Metabolism: Strict anaerobe growing well in ordinary media with neutral or slightly alkaline reaction. Optimum temperature 35° (growth poor at 37°). Hemolysis produced on erythrocytes (human and horse). Types A & B are generally proteolytic; type C only slightly proteolytic. Optimum pH 7.4 to 8. Above 37° toxin formation is impeded, below 20° toxin formation stops.

Cultivation:

Agar: 4 day growth: flat irregular, greyish yellow, filamentous colonies with alternately smooth and granular surface, and indefinite fringed periphery.

Deep glucose agar shake: 4 days growth: colonies thin semi-opaque discs with bi-convex brownish centers, translucent edges. Abundant gas formation.

Blood agar plate(Horse): 3 days: Irregular, round, 2-3 mm. colonies with smooth center fimbriate periphery. Alpha type hemolysis.

Cooked Meat Mediums(Brain): 4 days: Abundent growth, turbidity, gas formation, brain digested and blackened. Butyric acid odor.

Broth: 4 days: Dense turbidity, rancid odor.



## Bio-chemical Reactions:

	"A"	"B"	"C"
Glucose	: AG	: AG	: AG
Maltose	: AG	: AG	: AG
Salicin	: AG	: AG -	: -
Glycerol	: AG /	: AG /	: AG -
Lactose	: -	: -	: -
Inositol	: -	: -	: AG
Indol	: -	: -	: -
Nitrates	: -	: -	: -
NH <sub>3</sub>	: /	: /	: /
H <sub>2</sub> S	: /	: /	: /
Meth.Red	: -	: -	: -
V.P.	: -	: -	: -
Litmus milk	Reduced and alkaline, no coagulation.		

Serology: Types A & B are identifiable by aggl. and toxicity tests.

Their toxin is specific only for type, the antitoxin of one is not neutralized by the toxin of another. Type C, forming another separate specific toxin, is distinguished chiefly by lack of proteolytic powers.

Resistance: The bacilli without spores are readily killed by heat and chemicals. Spores withstand dry heat of 180° for 15 - 30 minutes, moist heat at 100° for 3 - 5 hours. Toxin is destroyed by 80° C in 5 - 15 minutes.

Identification: (see "Bacterial Food Poisoning")

Description: Slightly curved rods with rounded ends often resembling a comma; occur singly, in S-shaped pairs, short chains or spirals; actively motile; grow readily aerobically on simple media at 37°C.; agar plate colony: 1-2 mm. diameter, greyish yellow, translucent, low convex, with smooth or finely granular glistening surface and an entire edge, butyrous consistency; broth growth: abundant, with powdery deposit, thick surface pellicle.

Identifying Characteristics:

- (a) Their power to grow on solid media which are so alkaline (pH 8.0 to 8.4) that other organisms cannot develop.
- (b) Their initial growth at surface of liquid media, while accompanying organisms grow throughout the liquid.
- (c) Cholera red reaction  $\neq$  (also given by two saprophytic species).
- (d) Indol  $\neq$ , M.R. -, V.P.  $\neq$ ; acid, no gas in glucose, levulose, galactose, maltose, mannite, and sucrose; lactose may become acid after 14 days; litmus milk alkaline at top, slightly acid at bottom, not coagulated, slowly peptonized; nitrites produced from nitrates.
- (e) Gelatin stab growth: good filiform growth, confluent at top, discrete below, funnel-shaped liquefaction, with thick yellowish-brown pellicle on surface.
- (f) Agglutination with cholera immune serum.

Cholera-like Vibrios: There are several classified and probably many unclassified vibrios isolated from feces or water and differentiated on serological and biochemical characteristics.

Examination of Clinical Material: Vibrio comma may be isolated from the stools or intestinal contents of cases or carriers, from contaminated water or foods and identified by microscopic, cultural and serological methods.

Specimen Collection:

- (a) The "rice water" stool of cases or the feces of carriers are transmitted without the addition of glycerol or other preservative.
- (b) Surface water transmitted in a sterile liter flask.

Microscopic: A presumptive diagnosis of suspected cases, not of carriers, may be quickly made by examining stained spreads of flakes of mucus from the "rice water" stool; stain by Gram's method and with dilute-carbol-fuchsin; If Gram-negative, comma-shaped organisms are present, examine a hanging drop preparation. Presumptive positive report may be made if large numbers of typical, actively motile, vibrios are found. This finding must then be confirmed by cultural and serological examination.

Cultural: Specimens of feces from suspected cases or carriers should be planted, using two or more loopfulls of intestinal mucus or liquid feces, with the least possible delay and incubated at 37°C.

- (a) Alkaline peptone water; pH 8-8.4 (several tubes)
- (b) Alkaline nutrient agar, pH 8-8.4
- (c) Dieudonne's agar

Water under test is placed in 100 cc. amounts in sterile flasks, to each flask is added 10 cc. of 10% peptone water. After 6-12 hours incubation at 37°C., transfer a portion of the surface growth to the three media above.





Description: Minute rods with many coccid cells; 0.5 by 0.5 to 2.0 microns; Gram-negative; do not show bipolar staining; all species pathogenic to man are non-motile; do not liquefy gelatin; and fail to ferment any carbohydrates.

Habitat: Strict parasites, invading animal tissue, producing infection of the genital tract, the mammary gland or the lymphatic tissues and the intestinal tract. Br. melitensis, Br. abortus and Br. suis, primarily infect goats, cows and hogs, respectively, causing abortion and systemic infection; infectious to other domesticated animals; may infect man causing undulant fever (brucellosis). The motile species, Br. bronchiseptica causes distemper in dogs; also causes acute infection in other animals; and rarely infects man.

Br. melitensis, Br. abortus and Br. suis.

Description: Gram-negative, non-motile coccobacilli as for genus; Br. melitensis and Br. suis grow aerobically, Br. abortus requires 10% CO<sub>2</sub> for initial isolation and early culture transplants; growth on all media is slow, grows best on glucose liver infusion agar with pH 6.6; 48 hour colonies on plate are small, circular, convex, amorphous, smooth, glistening and entire; agar cultures turn media brownish after 7 days. The three species are very closely related; may be separated with difficulty on basis of (1) CO<sub>2</sub> requirement for growth, (2) growth on media containing certain dyes, (3) H<sub>2</sub>S production and (4) agglutinin absorption tests.

Habitat: Found in blood, urine, feces, exudates and occasionally sputum and nasal drainage of human cases; also in milk, cheese and other dairy products from unpasteurized milk from infected animals.

Laboratory examination of clinical material:

1. Microscopic.— Indistinguishable morphologically. However, Gram-stained smears from pathological lesions should be examined for the small Gram-negative rods described above.

2. Cultural.— While the organisms may be found in the blood early in the disease and during febrile periods and in urine and milk specimens at irregular intervals, the percentage of positive cultures, even from proved cases, is low.

a. Obtain specimen consisting of 10-12 cc. of blood or 50 cc. of urine or milk. Other body foci such as contents of ovarian cyst, synovial fluid, or excised glands may also be subjected to cultural study.

b. Inoculate two flasks containing 100 cc. of veal infusion broth pH 6.6 with 5 cc. of blood, several loopfulls of sediment from catheterized urine specimen, or several loopfulls of sediment and of cream layer from milk. Also streak specimen on two infusion agar plates.

c. Incubate one set of media in incubator at 37°C. for growth of Br. melitensis and Br. suis; place other set of media in jar containing 10% CO<sub>2</sub> and incubate at 37°C. for Br. abortus.

d. Examine plates and Gram-stained films from broth after 24-48 hours and at frequent intervals thereafter for growth. Streak new plates from broth at least once per week, even if no evidence of growth is discernible. Observe cultures for at least 4 weeks before reporting as negative.

e. Identify any positive culture as belonging to this group by agglutination with antisera prepared against either Br. abortus, Br. melitensis or Br. suis.

Note: Although not usually required the species of young cultures can be determined by (1) agglutinin absorption tests, (2) tests for H<sub>2</sub>S production, and (3) ability of the organism to grow on media containing certain dyes (basic fuchsin and thionin).

Table. - Differential characters of the three related species of Genus Brucella.

	10% CO <sub>2</sub> required for Primary isolation	H <sub>2</sub> S formation: (days).	Growth on media containing Thionin	Basic fuchsin
<u>Br. melitensis</u>	0	1	+++	+++
<u>Br. abortus</u>	++	2	0	+++
<u>Br. suis</u>	0	4	+++	0

3. Animal inoculations. - Br. melitensis and Br. suis, and less constantly Br. abortus, may be isolated from infected material by subcutaneous inoculation into guinea pigs (preferably males); after 4 weeks, kill the animal; examine Gram-stained smears from the lymph glands, spleen and liver; and make cultures from the liver, spleen, blood and lymph nodes. This test is seldom used because of the great danger of laboratory infection.

#### 4. Serological. -

##### a. Identification of pure cultures:

There is complete cross agglutination to titer between an antigen prepared with either species and antisera prepared against any other species. However, Br. abortus and Br. suis can be differentiated from Br. melitensis, but not from each other, by agglutinin absorption tests.

b. Serum from a patient taken after the fifth day of disease will usually contain agglutinins. Set up macroscopic agglutination tests in dilution of 1/20 to 1/640 or higher against a Brucella antigen (abortus, melitensis or suis) and against Pasteurella tularensis antigen. Agglutination of Brucella antigen in dilution of 1/100 or higher is considered to be significant. Cross agglutination in serum from patients with Brucellosis or tularemia is frequently present, but is less marked with the heterologous antigen. Agglutinins may persist for years after recovery. This is the most valuable test for diagnosing Brucella infection and is the only one routinely used.

/s



Pasteurella  
(Hemorrhagic septicaemia group)

Characteristics: Small Gram-negative rods showing bipolar staining. Aerobe, facultative anaerobe. Non-motile or motile. Frequently pathogenic, producing characteristic hemorrhagic infections in man and animals.

Includes:

- P. pestis - causing plague in man and rodents.
- P. tularensis - causing tularaemia in man and rodents.
- P. avicida )
- P. muricida ) associated with fowl cholera or hemorrhagic
- P. cuniculicida) septicaemia of birds or lower animals.

Pasteurella pestis

Habitat: A parasite of rats and other rodents, causes plague in man. Transmitted by the bite of infected rat flea, or by contact or contamination with rodent, or human case or carrier.

Characteristics: Short, thick bacillus; pleomorphic, especially in 3% salt agar; bipolar staining; grows readily on agar at 37°C. with raised, translucent, grayish-yellow, glistening, viscid growth.

May live for months in bodies of dead animals. Agglutinated by plague antiserum. Infectious by inoculation for small animals; subcutaneous injection into guinea pigs provokes local oedema followed by inflammatory swelling of regional lymph nodes, and a generalized infection to death in 2 - 5 days; postmortem appearance: glands enlarged, surrounded by hemorrhagic exudate; small grayish; necrotic areas in liver and spleen; bacilli found in local lesions, bubo, internal organs, especially spleen, and blood.

Collection of Specimens:

1. Pus or gland fluid from bubos, aspirated by syringe or collected after incision (may be forwarded to distant laboratories on agar slants).
2. Portions of affected tissues, removed at operation, to be forwarded in sterile bottles.
3. Blood specimens, taken during period of septicemia.
4. Autopsy materials, preferably bubo, lung, liver and spleen.
5. Sputum, in cases of pneumonic plague.
6. Rodent: The whole rodent, shipped in fruit preserving jar, sealed.

Microscopic Examination:

1. Stain films from suspect materials by Gram's method and methylene blue or dilute carbol-fuchsin (for bipolar staining).
2. The presence of typical Gram-negative, short, ovoid, polar-staining bacilli, including many degenerated and poorly stained forms, is suggestive but not conclusive evidence of P. pestis infection.

Culture:

1. Inoculate surface of blood agar, glycerol agar and 3% NaCl agar plates.
2. Plant blood specimen into nutrient broth and incubate before plating.
3. Incubate cultures at 30 to 35°C. for 36 to 48 hours.



4. Observe growth and transfer to agar, broth, litmus milk, gelatin, tryptone broth, lead acetate medium and sorbitol broth for further study ( see Chapter "Classification of Bacteria").

Agglutination: (Macroscopic method preferred, to avoid the spontaneous clumping confusing the microscopic test):

1. Make suspension of young agar culture in normal saline, using only the fine supernatant emulsion remaining after period of settling.
2. High-titre agglutinating serum (horse) is generally used.
3. Test is of greatest value in identifying suspect cultures, positive titre being interpreted in comparison with the titre of same serum tested with a known plague antigen.
4. Test is of little value as applied to patient's serum, for agglutinins do not appear in patients suffering from plague until about 9th day.
5. Salt solution controls are necessary in all tests to detect auto-agglutination.

Animal Inoculation:

1. Caution: Animals should be freed of all ecto-parasites, prior to use, by dipping in an antiseptic solution. Then place in glass jars covered with fine mesh gauze to prevent access or escape of any parasites. When handling animals, living or dead, protect the hands and arms by wearing rubber gloves and long sleeved gown.
2. Inoculate guinea pigs or mice subcutaneously with small amount of the original specimen or with a loopful of suspected culture. Putrefied materials may be applied to the freshly shaved abdomen of a guinea pig (plague bacilli penetrate the abraded skin, contaminants do not).
3. If P. pestis is present, the animals will develop characteristic lesions, die in 2 - 5 days with characteristic postmortem appearance; cultures of P. pestis may be isolated from the lesions.

Diagnosis of Plague in Rodents:

1. Postmortem appearance will usually evidence the natural infection in rodents.
  - a. Bubo, with hemorrhagic spots and areas of gray necrosis.
  - b. Subcutaneous and general congestion.
  - c. Granular liver, with punctate hemorrhage and grey-yellow spots.
  - d. Congested spleen.
  - e. Pleural effusion.
2. Bacilli may be found in bubo, liver, spleen and blood, and isolated from thence for study in pure culture by methods used for clinical materials.
3. Shipment to a distant laboratory for examination: The entire carcass is placed, without any preservative, in a tightly sealed container, which is packed in a second container to avoid breakage and escape of contents. The package must be shipped by express; federal laws prohibit the shipment of plague-infected materials by mail. Decomposition may be avoided by surrounding the inner container with ice or "dry ice". Label package "Perishable - for Bacteriological Examination - Please Expedite".

## Pasteurella tularensis

**Characteristics:** Small, Gram-negative, non-motile rods; pleomorphic, bacillary and coccoid forms; stained best with carbol-fuchsin and crystal violet, show bipolar staining; fail to grow on ordinary media; aerobic; require specially enriched media for growth; an organism which grows on plain agar or in broth is not P. tularensis; growth on serum-glucose-cystine agar, 2 to 5 days at 37°C.: minute, greyish-white colonies. Fairly susceptible to inimical agencies; killed by moist heat at 56°C. in 10 minutes. Agglutination tests of great value in diagnosis of disease by serum study, or in identification of cultures; agglutinins may persist for 20 years after recovery and a positive serum agglutination does not necessarily mean active infection. P. tularensis antiserum also agglutinates Brucella antigens to about  $\frac{1}{4}$  of its titre. P. tularensis is the cause of "tularemia", a plague-like infection of rodents, especially rabbits, and occasionally in man. Generally transmitted from rodents to man by infected blood-sucking insects, such as flies, ticks, lice, fleas and bedbug, or by direct handling of infected rabbits or squirrels. Accidental laboratory infections occur due to its ability to invade unbroken skin.

### Microscopic Examination:

Of value (1) to study morphology of organisms and (2) to rule out M. tuberculosis by observing acid-fast stain of spreads made from pathological materials.

### Culture:

1. Piece of infected tissue, pus, fluid or blood is planted on slants of glucose-cystine agar or blood cystine agar. Incubate at 37°C. for 3 to 5 days.
2. Blood agar plates also are planted to detect other organisms.
3. Observe cystine slants for characteristic colonies. If negative, continue observation for 21 days; if growth occurs, identify organism by stained spread, pure culture transplants and macroscopic agglutination tests with high titre immune serum.
4. Cultures made from blood and lesions of man are usually unsatisfactory. Cultures should be made from heart's blood, spleen, lymph nodes and liver of guinea pigs following inoculation with material from patient.

### Animal Inoculation:

1. Inoculate guinea pigs, rabbits, or mice with suspected materials from glands, ulcers or blood: (a) subcutaneously and (b) rubbed on the recently shaven, abraded abdomen if other bacteria are present.
2. Result: Death in 5 to 10 days (generally) with characteristic lesions:
  - a. At site of inoculation, hemorrhagic oedema, no pus.
  - b. Bubos, cervical, axillary or inguinal.
  - c. Glands enlarged and filled with dry, yellow, caseous material.
  - d. Spleen enlarged dark.
  - e. Liver contains discrete, white caseous granules.
  - f. Organisms can be seen in spreads and be cultured from spleen, liver, bubo and blood.



Agglutination Reaction: Macroscopic tube method preferred.

1. Set up agglutination tests of patient's serum against P. tularensis and Brucella (abortus or melitensis) antigens. Incubate in water bath at 45 - 55°C. for 12 to 18 hours.

2. Agglutination of P. tularensis by serum in dilutions of 1 to 80 or higher is considered diagnostic of tularemia, provided there is no cross agglutination with Brucella. Agglutinins appear in the patient's blood after the first week of the disease and usually increase rapidly.

3. Identity of a suspect culture may be established by a similar test using a suspension of the organisms and serial dilutions of a P. tularensis antiserum of known titre. The resultant agglutination to be significant, must be present in dilutions approaching the known titre of the serum.

A.M.S. 12/26/40



Characteristics: Minute rods, sometimes almost coccoid, sometimes thread-like and pleomorphic; Gram-negative; not acid-fast; non-motile, non-sporing, non-encapsulated. Strict parasites, do not grow on common media, require for their cultivation accessory substances present in the blood and fresh vegetable tissue. H. influenzae, H. ducreyi and H. pertussis are the three most important species.

Hemophilus influenzae

Characteristics: Very small, short rod; stains faintly, best by dilute carbolfuchsin or Giemsa stain; grows best on media containing hemoglobin, subcultures on plain or serum agar fail to grow; chocolate agar plate colony, 24 hours at 37°C.: small, pinpoint, transparent, smooth, raised; tendency to grow best near colony of other aerobic organism, i.e., "Satellite" colonies; not subject to agglutination test; does not have conspicuous biochemical activities.

Habitat: Commonly found in cultures of upper respiratory tract, their significance there questionable; probably not as name implies, related to the disease influenza. Occasionally found in pathological spinal fluids. "Koch-Weeks" bacillus, formerly called H. conjunctivitis, found in eye fluids in acute infectious conjunctivitis ("Pink eye"), is now classified as H. influenzae.

Identification:

1. Koch-Weeks bacillus:

(a) Make slide spread from conjunctiva, stain by Gram's method and with dilute carbolfuchsin.

(b) Observe for minute Gram-negative bacilli; often intracellular.

(c) Culture is not informative except to reveal other organisms.

2. Spinal fluid, respiratory tract and other suspect materials:

(a) Make culture on chocolate agar, incubate at 37°C. for 2 days.

(b) Suspect colonies are identified on colony appearance, microscopic morphology of organisms and failure of subcultures to grow on plain agar. Colony may be confused with a streptococcus colony.

(c) Specific identification considers source of the specimen, hemolytic properties and requirements of accessory growth factors.

Hemophilus pertussis

Characteristics: Like H. influenzae except bacilli are more uniform in size, with less pleomorphism, ferment no carbohydrates and do not require accessory factors for growth, but cannot be distinguished on morphology alone; colony on potato - glycerin-blood medium (pH 5.0) at 37°C. barely visible in 24 hours, plainly visible after 48-72 hours as small, greyish, raised, pearl-like growth; after several generations growth is freer, greyish, glistening, becoming in a few days heavy, almost like the growth of typhoid bacilli; then transplants will grow on plain agar.

Habitat: Constantly present in the respiratory secretions of whooping cough.

Identification: Cough plate method for isolating H. pertussis is preferable to sputum cultures: Open Petri dish, containing potato-glycerin-blood medium, is held in front of the mouth during a cough paroxysm. The organisms, sprayed on the plate with droplets of secretion appear in colonies after 37°C. for 48 hours. Colonies are larger, more opaque and whiter than those of H. influenzae.

### Hemophilus duplex

(Morax-Axenfeld bacillus)

Characteristics: Short stumpy, moderate size bacillus; often in diplo-form and chains. Cultivated only on media containing blood, serum or ascitic fluid. On Loeffler's blood slant colonies appear after 24-36 hours at 37°C. as small indentations which indicate a liquefaction of the medium.

Habitat: Found in eye in subacute infectious conjunctivitis. Not pathogenic for animals.

#### Identification:

1. Prepare slide spreads from conjunctival sac, stain with dilute carbol-fuchsin or Gram stain.
2. Short, stumpy bacilli in direct spreads are presumptively Morax-Axenfeld bacilli.
3. Culture on Loeffler's blood slant or other special media may confirm.

### Hemophilus ducreyi

Characteristics: Very small ovoid rod, non-motile, tendency to be in short chains and parallel rows; Gram-negative; tendency to be more deeply stained at the poles. In pus, the bacilli are often found within leucocytes. Difficult to cultivate; coagulated blood which has been kept for several days in sterile tubes (fresh blood will not do unless heated to 55°C. for 15 minutes) has been found to be a favorable medium.

Habitat: The cause of chancroid, the soft chancre, and found in the pus of ulcerating chancroidal ulcers, mixed with secondary infection, and in purer state in the chancroidal bubo. Not inoculable to lower animals.

#### Identification:

1. Examination of spreads or cultures for H. ducreyi is seldom practiced because of the technical difficulties of identification and the fact that chancroid lesions are usually distinguishable as such without laboratory confirmation.
2. Direct diagnostic cultivation from chancroidal lesion:
  - (a) Media: 1 cc. of sterile rabbit blood (freshly drawn) is placed in each of several small tubes, allowed to clot, then heated to 55°C. for 15 minutes and kept in icebox until used.
  - (b) Thoroughly cleanse lesion with sterile water or salt solution.
  - (c) Scrape material from bottom of ulcer or from beneath its edges, with a stiff platinum loop and plant in a tube of clotted blood by passing the wire around the clot.
  - (d) After 37°C. for 24 hours, the serum around the clot is stirred with the platinum loop and a spread is made and examined by Gram method.



(e) Characteristic chains of Gram-negative bacilli, sometimes in pure, sometimes in mixed culture, will sufficiently identify the organism.

(f) Transfer onto soft moist blood agar of pH 7.2 may give in 48 hours pinhead size, transparent, grey colonies with a firm, finely granular consistency.

3. Culture from unruptured bubo: pus is withdrawn by aspiration with a sterile hypodermic syringe and needle; cultured as above.

A.M.S. 12/18/40.





A war wound, in fact any traumatic wound, presents problems quite different from those encountered in aseptic surgery.

(1) Bacteria, aerobic and anaerobic, are carried by the projectiles or secondary missiles from the soil, clothing, or skin, into the tissues.

(2) Tissues are destroyed to a variable degree and such dead or devitalized tissues furnish excellent culture media for bacterial growth.

(3) Latent period, or interval, between contamination of the wound and definitive surgical aid, if beyond 6 hours, permits proliferation and penetration of the contaminating organisms.

The immediate aim of treatment, to be followed by such surgical restoration as indicated, is the prevention or limitation of infection. Bacteriological control, by examination of wound smears and cultures, determines these procedures.

Debridement is the first step by the surgeon in limiting infection in such wounds, by removing all the devitalized tissues and foreign bodies which would provide nidus of infection. Bacteria are greatly diminished in number but not eradicated by this procedure; at least most of the culture materials for bacterial growth are removed.

Smear or culture control is used in the follow-up programs for such wounds:

(1) The Smear method is simple and can be carried out by an average technician without elaborate procedures and yields results of value: smears are made of the wound secretions every other day, or daily as the time of secondary closure occurs, in such a way that an approximate estimate of the number of bacteria contained in the wound can be made. The examination need not begin earlier than 12 hours after the infliction of the wound, since up to that time few bacteria will be found. Smears are taken with a platinum loop from different parts of the wound, choosing points most likely to harbor bacteria, such as crevices, necrotic bone, foreign bodies, or deep sinuses; do not take from bleeding points, from smooth muscle or clean areas, and also avoid the skin adjacent to the wound. With a small platinum loop, small amounts of secretion are picked up and smeared upon slides in such a way that about the same area is covered by the different loopfuls of secretions - with practice a uniformity of technic is attained to provide comparable bacteria counts. These smears are allowed to dry, stained by Gram's method and examined under the microscope to gain an approximate estimate of the number and relative proportions of various types of organisms.

The number of bacteria per field (oil immersion objective) are counted. If the average number exceeds 50 or more to the field, more accurate counting is not necessary for the wound still contains too many bacteria to warrant

closure or relaxation of local therapy. Gradually as the wound improves, fewer and fewer bacteria will appear in the daily series of slides, and, when the number has dropped below 50 per field, careful counting may give an index to daily variation. Eventually the number will decrease to only one micro-organism per 5, 10, or 20 fields. The daily counts may be charted to provide a curve which will show the surgeon at a glance the numerical progress of the bacterial infection.

No smears are taken while hemorrhage exists; Dakin's, or other antiseptic fluids, are withheld for at least two hours before the smears are taken. When no bacteria can be found on smear, it does not mean that the wound is completely sterile; cultures may yet reveal organisms; and, when the period of secondary closure approaches, especially when streptococci have been present previously, cultures are to be taken, aimed particularly at the demonstration of hemolytic streptococci, before the secondary closure is done.

(2) Cultures are made at the beginning by planting the selected wound secretions onto fresh blood agar plates (without glucose). This is aimed at determining if pyogenic cocci, especially if hemolytic streptococci or staphylococci, are present. If the smears show a great many bacilli resembling the ordinary anaerobes, anaerobic cultures also may be planted. However, because of the long time required for working out the anaerobes in the laboratory, the surgeon is not concerned about this as a guide for his program of therapy. Suture of the wound is not carried out if hemolytic cocci of any kind are present, hence frequent culture upon blood plates are made during the progress of the treatment.

The surgeon's principles of procedure, considering these bacteriological problems are:

- 1) Debridement is done as soon as possible after infliction of the wound.
- 2) Primary suture is not done except in quiet periods of warfare and in hospitals where the patient may be retained for careful observations - otherwise, wound suture may lead to enclosure, in an imperfectly debrided wound, of harmful microorganisms, especially of the gas gangrene group.
- 3) Delayed primary suture may be done if the cultures, taken 18-48 hours after debridement, show no organisms; if hemolytic cocci are present, suture is not considered. The presence of one other organism per two fields (including a few anaerobes) does not counterindicate suture. Considerable number of organisms of any kind indicates delay of suture.
- 4) Secondary suture is undertaken when the organisms on two successive counts are few and the culture has shown an absence of haemolytic cocci.



GENUS CLOSTRIDIUM

Characteristics: Rods; anaerobic or microaerophilic; usually Gram +; form endospores in the so-called clostridium forms; often decompose proteins or ferment carbohydrates actively through the agency of enzymes; often parasitic and pathogenic.

Nomenclature: The war wound infections of World War I activated study of this group, adding many names to lists of those previously described, some known species being described under several different names. In the past two decades, this group has been further studied and classified, the genus Clostridium now including 51 described species, those of greatest importance in human medicine being Cl. tetani, Cl. paratubulinum, Cl. botulinum and the gas gangrene group elsewhere described.

CL. TETANI

Habitat: The tetanus bacillus is found in the intestinal tract of man and animals, in cultivated soils, road dirt, etc., and is therefore potentially present in wounds contaminated with these materials. Being a strict anaerobe, it tends to grow in the tissues as it does on culture media, in the depths remote from oxygen - therefore it will seldom infect surface wounds; will infect deep punctured or lacerated wounds. It produces in the tissues, as it does in the culture media, a powerful toxin which, on dissemination, produces the clinical features of Tetanus. This is strictly a local infection which becomes a general intoxication. The microorganisms can be found only in the local site, not in the blood stream or secretions.

Morphology and Staining: Slender, moderate-sized rods having round terminal spores, thicker than the cell, giving "drum-stick" appearance. No capsule. Slightly motile. Stain readily and are moderately Gram-positive, but even young cultures may have many Gram-negative forms.

Cultivation: Being a strict anaerobe, special facilities are required to attain growth. Grows well on ordinary media, growth greatly improved with blood or serum. Produces hemolysis of blood cells. Optimum temperature of growth 37° C. Optimum pH 7.4 - 7.8. Has slight proteolytic powers, gelatin slowly liquefied, coagulated albumin not liquefied. No acid or gas formed from carbohydrates. On litmus milk gives no change or slow precipitation of casein.

Colony Form: Irregularly round, 2 - 5 mm. in diameter, effuse, glistening, translucent colonies with irregularly granular surface, and ill-defined edge, showing filamentous, curled projections. Organisms tend to spread over entire surface of plate, making it difficult to obtain isolated colonies.

Resistance: Spores are very resistant to environmental influence; retain their vitality for years in a dried condition, resist boiling for 15 to 70 minutes, resist 5% Phenol or 1:1000 mercuric chloride for weeks. All spores are killed by exposure to dry heat, 160° C. for 1 hour or to steam under pressure, 120° C. for 20 minutes.

Serology: While Cl. tetani has been divided into 7 serological types, the toxin of all types is identical and is neutralized by antitoxin prepared from any member of the group.

Immunology: A powerful exotoxin is produced both in vivo and in vitro which is thermolabile, being destroyed by 65° C. in 5 minutes. An antitoxin, prepared from horse serum immunized by toxin injection, has long been in use and is of high proven value in prophylaxis and treatment of tetanus. Recently active immunizations against tetanus by the injection of tetanus toxoid has received favor and is often given, parallel with typhoid inoculations, as an aid to protection against war wound infection, or comparable peace time infections.

### EXAMINATIONS OF CLINICAL MATERIALS

The identification of Cl. tetani in infected wounds is usually quite difficult. While it may be demonstrated by microscopic or cultural methods, the most practical method available is animal inoculation.

Collection of Specimens: Pus and tissue fragments, taken from suspected wounds by surgical removal, on sterile cotton swab or on platinum loop, may be placed in a tube of sterile saline solution and this used for microscopic examination, cultures or toxicity tests. Spinal fluid from clinical cases may be tested for toxin content.

Microscopic Examination: Make film preparations of the suspected material. Stain and examine for the characteristic "drum-stick" spores of Cl. tetani. If present in small numbers, they may be overlooked. If non-virulent anaerobic or aerobic bacilli with round terminal spores are present, differentiation from the Cl. tetani cannot be made. Therefore this method of diagnosis is of very little practical value.

Cultural Examination: Inoculate the specimen into cooked meat media, into the water of condensation of an agar slant, and on blood agar plate; incubate these at 37° C. for 72 hours in anaerobic jar and observe for tetanus bacilli. The agar slant, so inoculated, may give a pure culture by the growth of an effuse, tenacious proteus-like growth over the surface of the slope; subcultures from the edge of this fern-like growth into the water of condensation of a fresh agar slant will yield the Cl. tetani in pure culture after several transfers. If spores are present in the cooked meat medium, heat the culture to 80° C. for 30 minutes to kill any nonsporing organisms and



then inoculate blood agar plates for the isolation of pure colonies.  
Cl. tetani cultures attain a foul odor resembling burnt horn.

#### DEMONSTRATION OF VIRULENT CL. TETANI

Animal Inoculation: Mix a portion of the original material, of the heated culture or, preferably, of a broth suspension of a pure culture, with sterile emory dust and inject 1.0 cc. subcutaneously into the thigh of a guinea-pig. A control pig receives the same injection plus an intraperitoneal inoculation of tetanus antitoxin. If Cl. tetani is present, the unprotected animal will develop tetanus and die in 1 to 4 days.

#### DEMONSTRATION OF TETANUS TOXIN

Inject subcutaneously, .5 cc. of filtrate of 10 day broth culture into each of two mice or guinea pigs, one of which has been given a prophylactic dose of antitoxin (intraperitoneal). Similar toxicity tests may be made by injecting animals with spinal fluid from cases of tetanus.

#### ORGANISMS ASSOCIATED WITH GAS GANGRENE

The anaerobic organisms associated with gas gangrene may be divided, on the basis of pathogenicity, into three groups:

- |                                  |  |
|----------------------------------|--|
| 1) Pathogenic <u>a)</u> for man: | <u>Cl. perfringens</u> (welchii)                 |
|                                  | <u>Cl. septicum</u> (vibrion septique)           |
|                                  | <u>Cl. novyi</u> (oedematiens)                   |
|                                  | <u>Cl. bifermentans</u> (oedematoides; sordelli) |
| <u>b)</u> for animals:           | <u>Cl. chauvoei</u>                              |
| 2) Lesser pathogenicity          | <u>Cl. histolyticum</u>                          |
|                                  | <u>Cl. fallax</u>                                |
| 3) Non-pathogenic                | <u>Cl. sporogenes</u>                            |
|                                  | <u>Cl. aerofoetidum</u>                          |
|                                  | <u>Cl. lento-putrescens</u> (putrificum)         |
|                                  | <u>Cl. tertium</u> and others.                   |

On the basis of their biochemical reactions, they may be separated into a saccharolytic group and a proteolytic group. There is not a strict demarcation of these properties, for most members have some properties of the other group,



that is some are both saccharolytic and proteolytic but are so classified as to the property which is most prominent. It may be noted that most of the pathogenic group are saccharolytic, most of the proteolytic are non-pathogenic (except Cl. histolyticum). The organisms of the proteolytic group are not in themselves pathogenic but complicate wounds by their intense proteolytic action; they are saprophytes, have no power of invading the tissues and, if present without members of the saccharolytic group, usually do not interfere with the healing of the wound.

CLOSTRIDIUM PERFRINGENS  
(Cl. welchii)

Description: Short, thick, non-motile, Gram + rods with rounded ends, moderate size, occur singly, in pairs, seldom in chains; form capsules in the animal body, at times in culture media; spores are large, oval, central or subterminal, formed only in alkaline sugar-free media and not in animal tissues; rods not distinctly swollen at sporulation; spores resist heating to 80° C. for one hour; grows best anaerobically, some growth microaerophilically; blood agar colony: round, domed, grey-white with smooth glistening surface, surrounded by zone of beta hemolysis; ferments all common sugars with production of large amount of gas, and lactic and butyric acids; latter giving characteristic odor; produces at least 4 different types (A, B, C, and D) of thermolabile exotoxin, sometimes all four being produced by one strain; each type of antitoxin is neutralizable by a specific antitoxin; type A is usually utilized for preparation of therapeutic antitoxin which may not protect against types B, C, or D, if present in the wounds; pathogenic for man and small animals, latter dying in a day following I.M. injection, with extensive blood stained fluid necrosis of tissue, marked gas formation; the muscles friable, pale pink color, the wound giving a foul acid odor, but there is no putrefaction.

Identifying Characteristics:

1. "Stormy fermentation" of milk.
2. Morphology and culture features.
3. Intravenous inoculation of rabbit (Welch-Nuttall test).
4. Guinea pig protection test.

General Comment: The free fermentation of sugars is a prominent characteristic both in its production of gas gangrene and the laboratory identification of cultures. Cl. perfringens ferments the muscle sugars, producing gas in the tissues; this is forced along fascial planes and vessels; hence it is called the "gas bacillus", giving the crepitation of gas gangrene. Fermentation in the test tube may be so marked as to blow out the plugs; in milk cultures it is made evident by the "stormy fermentation", an acid clot torn by gas bubbles, and separation of the milk into coagulum and whey.

Gas gangrene is essentially a local infection, not invading blood stream until shortly before death. Spores are never formed in the animal body.

The toxin produced is an exotoxin comparable to that of tetanus and diphtheria. An effective antitoxin is used in therapy. This antitoxin is specific only for Cl. perfringens, not for other wound anaerobes; therefore, if gas gangrene associated with Cl. perfringens and Cl. novyi is treated by a monovalent antitoxin, the latter infection would not be influenced. However, most commercial antitoxins are polyvalent, affording protection against all forms of gas gangrene.

CLOSTRIDIUM NOVI  
(Cl. oedematiens)

This is a large Gram-positive, sluggishly motile, spore bearing, anaerobic bacillus, resembling Anthrax in appearance; spores central, excentric to sub-terminal; rods distinctly swollen at sporulation. The lesion in an experimentally infected guinea pig is characterized by a whitish gelatinous exudate, little necrosis and absence of gas. It is feebly hemolytic, much less so than Welchii. It forms a soluble toxin by which antitoxic serum may be prepared.

CLOSTRIDIUM SEPTICUM  
(Vibron septicum of Pasteur)

This organism differs from previous two in that the rods are more slender and more pleomorphic. Even in young cultures, clubbed, citron, or navicular rods and filaments are present. It is motile, non-encapsulated, a strict anaerobe, and hemolytic. It invades the blood stream, producing a septicæmia. The occurrence of long filamentous forms in the livers of guinea pigs dying of this infection is characteristic and is used in identification of this organism. A powerful, soluble toxin is produced, which provokes local necrosis, not death, in guinea pigs inoculated intramuscularly, their death on intravenous inoculation. The antitoxin is specific, does not protect against Cl. perfringens or Cl. novyi.

This bacillus is closely related and very similar to Cl. chauvoei, the bacillus of symptomatic anthrax or blackleg of cattle and sheep. The latter has never been isolated from wound cultures and has never been known to cause infection in man.

CLOSTRIDIUM BIFERMENTANS  
(Cl. oedematoïdes and B. sordidli)

This species consists of large, sluggishly motile, Gram-positive bacilli; oval spores are formed centrally or subterminally, without swelling of bacillus. In pathogenicity it resembles closely Cl. novyi; however,



different strains show varying degrees of virulence and toxicity, from acute to none; the more toxic and virulent strains are commonly referred to as Bacillus sordelli.

#### PROTEOLYTIC GROUP

The organisms of this group can never produce gas gangrene without the presence of one or more bacilli of the saccharolytic group. They digest milk without the formation of a clot, liquefy and often blacken coagulated serum. These characteristics, plus the resultant offensive odor, point to their recognition. None of these organisms are very pathogenic or produce a general picture of toxemia in spite of the great liquefaction of tissue caused by them.

Cultures from wounds may contain both groups of bacilli, with difficulty of separation by culture methods. Such a mixed culture may be purified by animal inoculation, when the more pathogenic organisms of the saccharolytic group may invade the blood stream and be isolated from the heart's blood. This group is a great nuisance to the bacteriologist, for they give great difficulty in separation from the more pathogenic clostridia. Cl. sporogenes, the most frequent and active one, also gives confusion by resembling *Vibrio septique* morphologically.

Cl. sporogenes, next to Cl. perfringens, the anaerobe most frequently found in wound culture, is usually responsible for the foul odor of wounds, but its pathogenicity is negligible. It does not produce a soluble toxin and is not pathogenic for laboratory animals.

Cl. histolyticum differs distinctly from Cl. sporogenes in that it is more actively proteolytic, digesting living tissues. Injection of a pure culture I.M. into guinea pig results in rapid complete destruction of the skin and muscle, may expose the bone, the striking and characteristic feature being that in spite of the great local lesion the animal may remain well. The exudate will contain no gas, no putrid odor. A soluble toxin has been reported, and antitoxin prepared.

#### THE ANAEROBIC COCCI

Charts for the species identification of the anaerobic cocci are given here. They are fairly common contaminants of wounds and are not generally described in bacteriology texts. The aerobic streptococci are not here described in detail, for they are better known and are described in standard texts. Isolate pure cultures by selecting isolated colonies from anaerobic blood plates and identify by planting on the following media:

Infusion broth: examine for turbidity, sediment, gas, odor.\*  
 10% serum broth: examine for gas and odor.  
 Veillon's semi-solid agar: examine for gas.  
 Blood Agar plates: examine surface colonies for morphology,  
 action on blood.



Neopeptone water: examine for character of growth, gas production.

Litmus milk: examine for acid production, coagulation, clot retraction.

Gelatin: examine for liquefaction.

Carbohydrates: (1% dextrose, levulose, galactose, sucrose, maltose, lactose, and mannite, in sugar free base). Test media after 48 hours anaerobic cultivation for acid or acid and gas. Continue negatives up to 21 days.

Guinea Pig ) for pathogenicity.  
Mice )

### Key of ANAEROBIC NON-HEMOLYTIC STREPTOCOCCI

#### A. Strict Anaerobes:

##### I. Gas and fetid odor produced.

##### (a) No general turbidity in broth.

- |                          |                                    |
|--------------------------|------------------------------------|
| (1) Acid from maltose    | 1. <u>Streptococcus anaerobius</u> |
| (2) No acid from maltose | 2. <u>" foetidus</u>               |

##### (b) Turbidity in broth

- |   |                                     |
|---|-------------------------------------|
| (1) No gas in semi-solid agar (Veillon) |                                     |
| No gas in peptone water                 | 3. <u>Streptococcus putridus</u>    |
| (2) Gas in semi-solid agar              |                                     |
| Gas in peptone water                    | 4. <u>Streptococcus lanceolatus</u> |

##### II. No gas, no fetid odor produced.

##### (a) Milk not coagulated.

5. Streptococcus micros

##### (b) Milk coagulated.

- |                                   |                                     |
|-----------------------------------|-------------------------------------|
| (1) Viscous sediment in broth.    |                                     |
| Colonies on semi-solid agar       |                                     |
| blacken with age.                 | 6. <u>Streptococcus parvulus</u>    |
| (2) No viscous sediment in broth. |                                     |
| Colonies do not blacken with      |                                     |
| age.                              | 7. <u>Streptococcus intermedius</u> |

#### B. Microaerophilic

Strictly anaerobic on isolation.

Later microaerophilic

8. Streptococcus evolutus

WOUND BACTERIOLOGY RECORD

The cultured microorganism from debridement tissue and post operative control specimens may be placed in one of the following groups; by so recording it, there may result a uniformity of record for comparison and for tabulation of results of series of cases:

- |   |  |
|---|--|
| <p>1. <b>Aerobic Cocci:</b></p> <ul style="list-style-type: none"> <li>(a) Streptococcus-Beta (Hemolytic)</li> <li>(b) Streptococcus-Alpha (Viridans)</li> <li>(c) Streptococcus-Gamma (Anhemolytic)</li> <li>(d) <u>Staphylococcus aureus</u></li> <li>(e) <u>Staphylococcus albus</u></li> <li>(f) <u>Pneumococcus</u> (specify type)</li> <li>(g) Other cocci (specify)</li> </ul>                   | <p>2. <b>Anaerobic Cocci:</b></p> <ul style="list-style-type: none"> <li>(a) Streptococcus, hemolytic, strict anaerobe.</li> <li>(b) Streptococcus, hemolytic, microaerophilic.</li> <li>(c) Streptococcus, non-hemolytic gas forming.</li> <li>(d) Streptococcus, non-hemolytic non-gas forming.</li> <li>(e) Streptococcus, non-hemolytic microaerophilic.</li> <li>(f) Other anaerobic cocci (specify)</li> </ul> |
| <p>3. <b>Aerobic Bacilli:</b></p> <ul style="list-style-type: none"> <li>(a) Gram-positive, sporulating.</li> <li>(b) Gram-positive, non-sporulating.</li> <li>(c) Gram-negative, sporulating.</li> <li>(d) Escherichia group.</li> <li>(e) Aerobacter group.</li> <li>(f) Proteus group.</li> <li>(g) Pyocyaneus group.</li> <li>(h) Typhoid-dysentery group.</li> <li>(i) Others (specify)</li> </ul> | <p>4. <b>Anaerobic Bacilli:</b></p> <ul style="list-style-type: none"> <li>(a) <u>Clostridium perfringens</u>, (Welch)</li> <li>(b) <u>Clostridium septicum</u>, (vibrio septique)</li> <li>(c) <u>Clostridium novyi</u>, (oedematiens)</li> <li>(d) <u>Clostridium histolyticum</u>.</li> <li>(e) <u>Clostridium tetani</u>.</li> <li>(f) Others (specify)</li> </ul>   |

BACTERIOLOGICAL INVESTIGATION OF WAR WOUNDS\*Purpose:

1. To provide more precise information about infections so often associated with war wounds, especially the anaerobic infections.
2. To supplement clinical data as to the effects of various methods of treatment upon these infections.
3. To secure further data as to the importance of cross infection by streptococci (and other organisms) in hospital wards; and as to how these may be controlled.

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\*Reference: "Notes on the Diagnosis and Treatment of Gas Gangrene" H. M. Stationery Office, London, 1940, including scheme suggested by Committee of London, Sector Pathologists.



Method of Collection of specimens:

1. Swab: mounted on wooden applicator, 5 inches long, wholly encased in a sterile tube plugged with cotton.
2. At the operation an attendant can remove the plug and tip out the end of the swab stick, thus allowing the surgeon to take the specimen and replace it into the tube without impairing his sterility. The attendant will then plug the tube, label it and dispatch it to the laboratory.
3. Pus may be collected to some advantage, in a test tube or a capillary pipette, as this allows of a more satisfactory microscopical examination.

4. Specimens should be taken from the deepest parts of a wound.

Time of Collection of Specimens: the following program will enable the course of the infection to be ascertained and the incidence of hospital infection to be determined.

1. Initial specimens should be collected at the commencement of the operation, in order to ascertain the nature of the primary infection.
2. Another specimen should be taken at the time of the first complete dressing.
3. Further specimens should be taken at weekly intervals.
4. Specimens should be taken more frequently in cases receiving special methods of treatment (such as by the sulfonamide drugs) and the treatment checked by as detailed bacteriological examination as possible, following the methods outlined below.

Procedure:

1. Microscopic examination of films of material from the wound:  
Gram-stained films should always be examined. They may give immediate information to the surgeon as to the general nature of the infection and also indicate to the bacteriologist suitable special methods of culture.
2. Cultural Methods. It is recommended that the following cultures be made:
  - (a) Blood agar plate of unheated pus; for aerobic incubation.
  - (b) Blood agar plate of unheated pus, for cultivation in anaerobic jar. These anaerobic plates should be well dried before incubation, to prevent the undue spreading of colonies. An indicator tube should always be placed in the anaerobic jar.
  - (c) Litmus milk for the detection of Cl. perfringens. The milk tubes should be boiled to expel the air; and, on cooling, they should be thickly inoculated from the swab or pus, covered with melted vaseline and incubated without further anaerobic precautions.
  - (d) Robertson's meat medium: This grows both aerobes and anaerobes, and is especially useful in that it provides a culture to which the bacteriologist can return if, for any reason, his plate cultures are unsuccessful. It also allows slowly developing anaerobes to be detected in subcultures made after some days incubation. These cultures, if they are to be stored, should be covered with a layer of liquid paraffin to prevent drying.



- (e) If, in any laboratory, it is impossible to proceed with the isolation of the anaerobes, a meat tube should be inoculated from the wound swab and sent to a reference laboratory.

#### Observation of Anaerobic Plate Cultures:

(1) These will yield many aerobic organisms, both spore-bearers and non-spore-bearers, in addition to the obligate anaerobes. Some strains of bacteria which first appear exclusively on the anaerobic plate will prove on subculture to be aerobes. Colonies of some aerobic spore bearers, when growing anaerobically, will often simulate those of Clostridia.

(2) The cultures should be examined the day after inoculation and again after 48 hours, as the colonies of the spore-bearing anaerobes may then be more characteristic.

#### (3) Spore-bearing Anaerobic bacteria

Organism	Surface colonies	Deep colonies in agar	Litmus milk	Coagulated serum or egg	Spores
<u>Cl. perfringens</u>	Large, regular probably haemolytic.	Lenticular, opaque.	Stormy acid clot*	—	Seldom seen in culture.
<u>Cl. novyi</u>	Clear, slightly irregular.	Woolly	Acid	—	Central and terminal
<u>Cl. septicum</u>	Transparent, spreading.	Transparent, branching.	Little change (? slight acid).	—	Central or sub-terminal
<u>Cl. sporogenes</u>	Medusa-head (if plate dry); irregular (if plate moist).	Woolly; opaque.	Digestion with foul odour.	Digestion	Central or sub-terminal
<u>Cl. tertium</u>	Small, clear, circular.	Small, lenticular.	Little change	—	Terminal oval.
<u>Cl. tetani</u>	Very transparent, spreading.	Delicate filaments.	Little change	—	Terminal round.

\* This appearance may be simulated by certain other microbes.

(4) It is emphasized that the presence of Cl. perfringens in a wound is not necessarily an indication of gas gangrene; this organism is present in the cavities of many wounds of patients not suffering from that disease.

(5) Fermentation tests may be made after a pure culture is attained. It should be noted that some indicators may be irreversibly decolorized in the anaerobic jar. Tubes showing no apparent increase in acidity after anaerobic incubation should be tested with a drop or two of fresh indicator.

(6) Streptococci may be found in anerobic cultures which do not appear on aerobic blood agar plates. While some of these are true anaerobes, many are cocci which, when just isolated, will not grow aerobically but which soon become acclimatized and grow readily in open tubes and plates.

(7) True anaerobic streptococci should be investigated as to cultural reactions, digestion of albumin, production of foetid odor, sugar reactions and as to pathogenicity for animals. Cultures may be maintained on Robertson's meat medium for future study. It is suggested that a microscopical examination of the original meat culture should be made after some 3 days incubation (this also gives valuable information as to the spore-bearing anaerobes present) and, if streptococci are seen which did not appear in the culture plates, further cultures should be made on blood-agar, anaerobically. Streptococcal colonies which appear may then be subcultured into "sloppy" glucose-agar (glucose-broth containing 0.1 - 0.2% agar).  
Aerobic Plate Cultures: Note and study as described elsewhere:

1. Hemolytic streptococci.
2. Staphylococci.
3. Other organisms.
4. Relative numbers of different bacteria present.  
 (as indicated by the primary microscopical examination and from the aerobic and anaerobic cultures).

#### Special Procedures:

##### 1. Potassium Tellurite media:

(a) The bacteriostatic properties of potassium tellurite on nutrient agar are approximately as follows:

Haemophilic Bacilli and most coliforms are inhibited by a conc. of 1:500,000.

Ps. aeruginosa is inhibited by a conc. of 1:50,000.

Proteus strains are variable and many are resistant to 1:50,000.

Streptococci, staphylococci and diphtheroid bacilli are resistant to 1:10,000.



(b) If, in the direct microscopical examination of the pus, large numbers of coliform organisms are seen, potassium tellurite may be incorporated in the medium in a concentration of about 1 in 50,000, in order to inhibit them and to allow the cocci to be easily isolated.

(c) A much simpler method is to inoculate a plate in the usual way and then to spread 2 or 3 drops of 1 in 1000 tellurite over half of the plate. In this way one half of the plate is an ordinary culture, while on the other half the coliforms are generally completely inhibited. A convenient stock solution is 1 in 1000.

(d) Potassium tellurite may be used in the same way for the separation of anaerobes as there are differences in the sensitivity of different members of this group to the chemical. More work remains to be done in order to determine its exact usefulness in this direction.

## 2. Isolation of Organisms from Material Containing Genus Proteus Organisms.

(a) Blood agar plate is inoculated in the usual way. Melted agar at 45° C. is poured over the surface of the plate to a depth of 2 or 3 mm. and allowed to set. After incubation it will be found that any colonies of Proteus, growing between the two layers of agar, show no tendency to spread and colonies of other organisms can be easily picked out from among them. Some of the Proteus may spread round the edge of the agar to the upper surface and this must be killed before any deep colonies are picked out. A satisfactory method is by flooding the surface of the plate with saturated mercuric chloride solution for about 30 seconds; the solution is washed off with tap water and some of the surface growth of Proteus is scraped off with the end of a microscope slide to enable the deep colonies to be picked out and subcultured.

(b) Potassium tellurite in concentration of 1:20,000 to 1:50,000 can be incorporated in the agar which is poured over the surface. This inhibits most of the coliform bacilli and sometimes prevents Proteus group organisms from spreading over the surface.

## 3. Negative Staining for the Detection of Clostridial Spores in Culture:

(a) A small drop of nigrosin solution is placed on a slide, and some of the culture is mixed with this and spread out into a thin film by means of a wire or another slide. This film is allowed to dry and can then be examined. In the thicker parts of the film the large spores stand out as clear spots, while the bacillary portions are partly overlaid by the nigrosin. This simple method of demonstrating spores may, if necessary, be confirmed by the usual staining methods.

(b) Alternative method: make a film of the bacteria in water on a slide in exactly the same way as is done preparatory to staining. When this film is dry a small drop of nigrosin is placed on the slide and is spread in a thin film over the bacteria.



(c) Nigrosin solution: Gurr's Nigrosin in water about three quarters saturated.

4. Influence of CO<sub>2</sub> on the Growth of Bacteria in Wounds:

(a) Some streptococci and other bacteria will not grow, or will grow only poorly, unless there is an increased amount of CO<sub>2</sub> in the atmosphere. Where facilities exist, observations may be made on the effect of an atmosphere of from 2 to 10% of CO<sub>2</sub> on the growth of aerobes and anaerobes of war wounds.

(b) Cultures, either plates or tubes, are placed in a container of about 3,600 cc. volume. There is placed in the container an open tube 8" x 1" containing 8 cc. (excess) of 25% HCl; a marble chip of about 0.7 gram is dropped into the acid and the lid pressed down; this will give about 5% CO<sub>2</sub> at 37° C.

5. Indicator Tube for Anaerobic Jar:

To a tube of 5 cc. of 2% glucose-broth add .1 cc. of Loeffler's alkaline methylene blue. This should become decolorized in the jar and it should remain colorless throughout incubation if anaerobic conditions are maintained.

A.M.S., 12/23/40.



Characteristics: Slender, undulating, corkscrew-like, flexible, filamentous organisms. They have short or long spirals with the twists in three dimensions. The number, depth and relative length, and sharpness of angle of spirals have diagnostic importance, though somewhat variable. Motile by sinuous, rotating movement of the body, not by flagella as in the case of bacteria. Stain with difficulty by ordinary stains though some (genus Borrelia) stain readily; the polychrome methylene blue stains of Wright and Giemsa are most used; silver impregnation method is applicable to the more resistant forms, Fontana stain for spreads, Levaditi stain for tissues. Most readily demonstrable, to reveal their characteristic motility, in the fresh state by dark ground illumination. Cultivation difficult and generally not practical. Animal inoculation significant in a few pathogenic species.

Habitat: Ubiquitous, occurring in nature in soil, water, decaying organic materials and on and in the bodies of man, animals and plants. Some are saprophytes, others are commensals, a few are pathogenic, causing such severe diseases as: syphilis, yaws, relapsing fever and infectious jaundice.

#### BORRELIA RECURRENTIS

(Relapsing fever spirochete)

Characteristics: Spirochetes having large, wavy, inconstant spirals, usually about 5; when seen under darkfield illumination, the organisms are very active, in length several times the diameter of an erythrocyte, rapidly progress in either direction, disturbing the red cells by their motion; stain readily and uniformly by polychrome stains (Wright's or Giemsa's) and by simple stains; difficult to cultivate; inoculable into mice and rats, there causing periodicity of spirochetemia but no demonstrable clinical symptoms or tissue lesions.

Habitat: The cause of Relapsing fever; found in blood and tissues of patients suffering from relapsing fever and in the body and intestinal contents of the infected vectors, ticks and lice. The name applies to the spirochete of European relapsing fever; a number of other species—names have been given for the spirochetes of the United States and Mexico (B. turicata), Central and South America (B. venezuelensis) and others, differentiation of which is based only on specific immunological reactions. Some lower animals may serve as reservoirs of infection, in the United States, the armadillo and the opossum.



Identification: Fresh or citrated blood, taken during febrile paroxysm, is examined:

(1) Darkfield illumination of fresh, thin slide-cover glass preparation, for the characteristic motility and morphology.

(2) Slide spread, stained by Giemsa's method or by dilute carbol-fuchsin for morphology. Here the forms are much distorted, the spirals often obliterated, so that the characteristic morphology cannot be found. These spirochetes may sometimes be detected and the diagnosis suggested, in a routine Wright's stain for differential blood count.

(3) White mouse or rat inoculation, intraperitoneal, of .2 to .5 cc of blood; examine fresh tail blood from the 2nd to 14th day for spirochetes.

### FUSOSPIROCHETAL DISEASE

(Vincent's Angina)

Definition: Vincent's angina is an inflammatory lesion in the mouth, pharynx or throat, most often affecting gum margins and tonsils. An acute inflammation may lead to the formation of a pseudomembrane, suggesting that of diphtheria; later there are punched out ulcers, suggestive of those of syphilis. The disease is localized, generally mild with minimal systemic disturbances. Two microorganisms are almost always found together, in great numbers, in films from these lesions; the two forms apparently living in symbiosis. They are rarely present alone, being usually accompanied by other microorganisms, such as staphylococci, streptococci, even diphtheria bacilli; the latter finding being more significant than the Vincent organisms alone.

Fusobacterium plauti-vincenti (fusiform bacillus of Vincent): Large bacilli, thick in middle, tapering toward ends to blunt or sharp points. Readily stained by Loeffler's methylene blue, carbol-fuchsin, or Giemsa stain, with characteristic inequality in the intensity of the stain, being more deeply stained near the end; banded alteration of stained or unstained areas in the central body, not unlike the metachromatic granules of diphtheria bacilli.

Borrelia vincentii: Spirochetes somewhat like those of relapsing fever, longer than the fusiform bacilli; made up of variable numbers of undulations, shallow and irregular in their curvatures, unlike the more regularly steep waves of Treponema pallidum. They stain more evenly and less distinctly than the fusiform bacilli.

Identification:

1. Make slide spreads from the ulcerative lesions, fix in flame and stain deeply with dilute carbol-fuchsin, crystal violet or Wright's stain and examine for fusiform bacilli and spirochetes.
2. Positive results will be evidenced by finding great numbers of both fusiform bacilli and spirochetes. A few forms of either type is not significant.

TREPONEMA PALLIDUM

Characteristics: Delicate spirochete coiled in 8 to 14 regular, rigid, sharp spirals; spirals equal or greater in depth than in length, with acute rather than obtuse angles. As seen under darkfield illumination, it appears as a highly refractile, long, slender, spiral; silvery form with serpentine, corkscrew-like movement; motile, but does not progress rapidly or far, motion rotational with undulations.

Made visible most effectively by darkfield illumination. Difficult to stain with aniline dyes other than the Giemsa stain; body stained pink by Giemsa stain or black by silver impregnation method, Fontana stain in spreads, Levaditi in tissues.

May be cultured by special methods and inoculated into some lower animals, neither procedure being practical for diagnostic purposes.

Habitat: Strict parasite of humans, causing the infectious disease syphilis, with protean manifestations; transmitted only by direct contact, generally through sexual intercourse, occasionally through intimate contact of other mucous membrane or skin sites. Syphilis, one of the most prevalent and important of all infectious diseases, usually progresses through a number of stages, irregular and varied:

1. Incubation period of 4 to 6 weeks. Spirochetes then cannot be demonstrated.

2. Primary stage: "Hard chancre" at site of inoculation. Starts as a papule, enlarges, becomes hardened and then ulcerates, forming an ulcer with a firm base and hard edge in typical form, but atypical lesions frequently occur, especially, if mixed with secondary infection or coexistent with chancroid. Spirochetes can be found in fluid expressed from this chancre. The spirochetes will not necessarily be on the surface, rather in the deeper tissues and in the serum exuding from the ulcerated lesion; at this stage they have already become disseminated to a general infection, can be demonstrated in fluid aspirated from satellite lymph gland, but cannot readily be found in the blood or other areas though potentially there.

3. Secondary stage: Characterized by mucous patches, skin rashes and a variety of superficial lesions. Treponema pallidum can usually be found in material from these lesions.



4. Tertiary stage with lesions of viscera, bones, central nervous and cardiovascular systems; tendency to deep rather than superficial lesions. Spirochetes are usually scanty, not readily demonstrated in these lesions.

Identification: (Different procedures applicable to different lesions and stages).

1. Darkfield Examination:

(a) Lesions are cleansed of surface crust, detritus, pus and surface organisms by gauze, or cotton applicator. If lesion has received any germicidal agent, examination is deferred until all germicide has been removed and the lesion has had applied to it, only a saline pack for a day or two.

(b) Primary lesions are then given some trauma, to provoke exudation of serum, by gently rolling the lesions between the gloved finger and thumb or by rubbing its surface with a dry cotton applicator; avoid hemorrhage (though a few erythrocytes or pus cells are desirable to aid in obtaining proper illumination).

(c) Secondary lesions are merely cleansed and abraded.

(d) Slide-cover glass, fresh preparation may be made from accessible lesions by merely touching the slide to tissue juice and immediately placing the cover glass over this moist drop. Vaseline placed around edge will prevent drying. If lesion is less accessible, the fluid may be collected in a capillary pipette and placed on slide from this.

(e) Examine immediately on darkfield microscope for characteristic morphology and motion of Treponema pallidum. Exercise caution not to misinterpret observation. There are many saprophytic spirochetes which are easily distinguished; there are a few spirochetes, especially in the mouth, which are more difficult to distinguish.

(f) "Artifact spirochetes" provoke mistakes to those unfamiliar with the appearance of blood, pus and cultures under darkfield illumination. Wavy filamentous structures may occur there which give a false impression of spirochetes; forms given off by red corpuscles in a drop under a cover glass, may falsely suggest spirochetes.

(g) Report findings with qualifying data, such as notation of location of lesion examined, the occurrence of conditions making examination unrepresentative, etc.

2. Delayed darkfield method: This is a scheme of forwarding lesion fluids to a distant laboratory for darkfield examination; employable when facilities for darkfield examination are not locally available, or when local examiners desire confirmation of their own findings by a consultant. A tissue fluid from a suspected lesion is allowed to flow into a capillary tube 8 cm. long by 1 mm. diameter; the two ends of this tube are sealed by pressing into a soft paraffin-vasoline mixture (50% of each) and these tubes



forwarded for the darkfield examination. At examining laboratory the serum may be transferred to a slide by pressing one end of the capillary tube into a paraffin-vaseline mixture until the opposite end plug is forced out.

3. Nigrosine method: This is not strictly a staining method, for it leaves the unstained spirochete in a black field. A loopful of the fresh tissue fluid is mixed with a loopful of 5% aqueous solution of nigrosine (plus .5% formalin as a preservative); this mixture is spread on a glass slide, dried and examined by ordinary illumination with oil immersion objective. A remote examination may be made, by forwarding an air dried drop of the exudate on a slide; the laboratory adds a loopful of water to this to dissolve the exudate and proceeds with the nigrosine preparation. Results are far inferior to the darkfield method, for the characteristic motility is absent and the spirochetes, by distortion, have lost much of their characteristic morphology.
4. India ink method: Like the nigrosine method, a drop of material is mixed with a drop of drawing ink and the mixture spread on a slide. When dry, examine for white spirals against a dark background.
5. Stained spread examination: By Giemsa or Fontana methods.
6. Local Wassermann: Considerable amount of serum is collected from the local lesion and used for complement fixation test.
7. Blood serum and spinal fluid serology: Applicable to later stages. It is customary to subject all venereal patients, even after repeated negative darkfields, to follow-up blood tests for several months.

#### LEPTOSPIRA ICTERHAEMORRHAGIAE

(Weil's Disease - Infectious Jaundice)

Characteristics: Spirochetes of many fine coils, so fine as to be difficult to distinguish; one or both ends may be bent into a hook. Rapid spinning motion with intermittent active lashings. Difficult to stain; stained reddish by Giemsa method. Cultivated only by special methods. Inoculable into guinea pig with distinctive lesions.

Habitat: The blood and kidneys of infected wild rats. The blood, urine, kidney, biliary tract of patients with infectious jaundice (Weil's Disease).

Identification:

1. Guinea pig inoculation: Inoculate 3 to 5 cc. of fresh blood, fresh urine sediment or tissue suspension, intraperitoneally into white guinea pig; observe it daily for fever, jaundice in the ears, eyes and about genitalia and for leptospira in the blood (usually found after the 4th day). After the animal dies, large numbers of leptospira can be demonstrated in emulsions of the liver, kidneys and adrenals.
2. Darkfield examination of tissue emulsions, occasionally of urine or biliary sediment, for the motile leptospira.
3. Stained spreads and cultures have limited application.



CORYNEBACTERIUM DIPHTHERIAE  
(*Diphtheria bacillus*)

Description: Slender rods, straight or slightly curved, of medium size; often lie at various angles to one another forming V or Y shapes, or clumped as Chinese letters; generally not uniform in thickness, exhibiting rounded, pointed or swollen ends or enlargements along the length of the cell; usually stain unevenly, showing barred and granular large forms, solid staining short forms; gram-positive, nonmotile; grow readily at 37°, preferably on Loeffler's serum or blood agar as small, circular, smooth, moist, grayish to creamy-white colonies, some strains giving narrow zone of hemolysis on blood agar; pathogenic to man and to guinea pigs.

Habitat: The cause of diphtheria, usually found in the mucous membranes of the nose, throat and larynx of cases and carriers, occasionally found as cause of conjunctivitis, wound infection, middle ear infection and bronchopneumonia. It is not found in the blood stream, the general symptoms being caused by the powerful toxin formed at the local site of infection.

Identifying Characteristics: (1) Shape, size, irregular staining, V or Y arrangements as seen in a direct spread or spread from Loeffler's medium and stained by Loeffler's methylene blue or Neisser's stain.

- (2) Growth freer on Loeffler's serum medium than that of other organisms.
- (3) Colony form on blood agar. Colony on tellurite medium becomes black.
- (4) Pathogenicity for guinea pig (see virulence test).
- (5) Carbohydrate fermentation (see chart below).

Collection and Transmission of Specimen for Examination: Cotton swab may be applied to the involved area (throat, nose, wound) or to the membrane or exudate from that area with care to gather a considerable amount of the exudate on the swab, with caution not to contaminate the swab by it touching the tongue or other noninvolved areas. Use this swab for:

- (a) Immediate inoculation of Loeffler's serum slant for 18-24 hours, incubation at 37°C., or for shipment to distant laboratory.
- (b) Immediate inoculation of blood agar plate for incubation at 37°, 24 hours.
- (c) Spread on slide for direct Neisser stain examination.
- (d) (Optional) plant on "tellurite media".

Microscopic Examination: (Direct spread, Neisser's or Loeffler's stain).

An immediate presumptive diagnosis can sometimes be made on the basis of morphology and staining features of what few diphtheria bacilli may be observed in the direct smear, but here they will be confusedly mixed with the many other microorganisms of mouth or wound flora. Vincent's organisms and diphtheroids may give confusion, should be noted on report if found. Negative finding by direct method cannot be given value. Presumptive positive finding should be confirmed by cultural and virulence tests.



Cultural Examination: (a) Loeffler's tube, after 18-24 hours incubation at 37°C, is examined by broad needle drag along its surface, this then spread on a slide and planted on blood agar and tellurite media for later pure colony isolation. The slide spread is stained by Neisser method and observed for diphtheria bacilli; the irregularity of staining and shape, the metachromatic granules may be noted. If typical diphtheria bacilli are found and the culture is from a suspected case, a presumptive diagnosis should be made at once. If the culture is from a suspected carrier, diphtheria-like bacilli should be further identified by fermentation and virulence test.

(b) Blood agar plate will provide information on general bacterial flora, particularly streptococci, and will give opportunity for notation of colony form and single colony isolation of diphtheria-like bacilli.

(c) Tellurite media will point out the diphtheria-like colonies by black color.

(d) (Optional - rapid) Apply a sterile serum-swab to involved area, return to serum-tube and incubate for a few hours; transfer to other media and examine slide made by gently rolling swab out into thin film. Sterile serum swabs are prepared by placing sterile swabs into sterile tubes containing a few cc. of serum. Some such swabs are made to contain 2% potassium tellurite to attain blackening from growth of diphtheria bacilli.

Fermentation Reactions: *C. diphtheriae* can be differentiated from related organisms by their fermentation reaction in dextrose, saccharose and dextrin. The absence of power of a particular organism to ferment glucose and its ability to ferment saccharose is usually sufficient to exclude the organism from being a diphtheria bacillus.

Virulence Test: This is the only certain method by which the identity and virulence of *C. diphtheriae* can be confirmed or distinguished from nonvirulent variants. No other known species of this genus occurring in man produces a fatal toxemia in guinea pigs. Pure cultures are preferred for this test, but for speedy test the suspension of a heavily positive Loeffler's tube may be substituted.

(a) Subcutaneous Method: Inject 2 cc. of a pure culture grown for 48 hours in infusion broth or 4 cc. of a Loeffler's slant suspension in 10 cc. of saline, subcutaneously into a 250 gram guinea pig. At the same time a similar injection of the culture is made into a control guinea pig which had been given 250 units of diphtheria antitoxin, intraperitoneally, 24 hours previously. If the organism is a virulent diphtheria bacillus the unprotected animal will die within 3 to 5 days and on post mortem will show a gelatinous edema around point of injection and enlarged hemorrhagic adrenals.

(b) Intracutaneous Method: Two guinea pigs of 250 grams are used, one of which has been injected intraperitoneally with 250 units of diphtheria antitoxin 24 hours previously. The growth from a 24 hour Loeffler's slant is suspended in 20 cc. of normal saline and .15 cc. is injected intracutaneously at corresponding site of each pig. Six cultures may be tested at the same time on two animals. Virulent strains of diphtheria bacilli produce a definitely circumscribed local infiltrated lesion which shows superficial necrosis in 2-3 days. In the control pig the skin remains normal. If a mixed culture was used for test, and contained streptococci or staphylococci with sufficient virulence, local lesions will occur in both animals; the test

would then be considered inconclusive and repeated using a pure culture.

Schick Test: This is an intracutaneous skin test to evidence the presence or absence of immunity to C. diphtheriae. The injection consists of .1cc. of diphtheria toxin (1/50 m.l.l.). A control test uses the same material which has been made inert by heat (75° for 5 minutes). Results are noted daily for 4 days and recorded as Positive, Negative, Positive Combined or Negative Combined reactions.

C. pseudodiphthericum (Hofmann's bacillus): This organism is shorter and thicker than C. diphtheriae, usually straight and clubbed at one end, rarely at both; when Loeffler stained it occasionally shows unstained transverse bands which, unlike those in C. diphtheriae, hardly ever exceed one or two. Sometimes the transverse band gives the bacillus a diplococcoid appearance; no polar bodies are demonstrable by Neisser's stain; it grows more luxuriantly, colonies larger, less transparent and whiter than are those of true diphtheria bacilli; a positive means of distinction is its inability to form acid on sugar media; it is not pathogenic to guinea pigs or to man. It is a common mouth commensal, may be found in 42% of normal throat cultures; diphtheria-like bacilli which prove to be avirulent generally are found to be C. pseudodiphthericum.

C. xerose (Xerosis bacillus): This is a harmless saprophyte, commonly found in the normal or inflamed conjunctiva of the eye. It closely resembles C. diphtheriae, and is indistinguishable morphologically and culturally though generally shorter; polar bodies may occasionally be seen; it differs in its acidifying action on sugar media and its nonpathogenicity to guinea pigs.

	Hiss Serum Water plus 1%:			Virulence:
	Dextrinose	Saccharose	Dextrin	
<u>C. diphtheriae</u>	+	-	+	Virulent
<u>C. pseudodiphtheriae</u>	-	-	-	Nonvirulent
<u>C. Xerose</u>	+	+	-	Nonvirulent

Diphtheroid Bacilli: There is a large group of ill defined organisms given this general name because of their morphological resemblance to the diphtheria bacillus; often show metachromatic granules; are not virulent when tested by the guinea pig virulence test. They are common saprophytes of the throat, skin and other body areas, are so ubiquitous that any association of them with specific disease must be avoided; they must be distinguished from virulent and therefore significant diphtheria bacilli.





MYCOBACTERIUM TUBERCULOSIS  
(Tubercle bacillus)

Description: Slender rods, straight or slightly curved with rounded ends; occur singly, in threads or in clumps; may stain evenly or irregularly showing granular, beaded or banded forms; stain with difficulty but when once stained are acid fast; growth on media slow, aerobic, aided by glycerine or other enrichments; growth on glycerine agar in 4 weeks at 37°; colonies minute, crumb-like, irregular, whitish-yellow, later brownish, ridged, becoming dry; pathogenic to guinea pig.

Habitat: A strict parasite, causing tuberculosis of man, cattle and other animals; human and bovine varieties are distinguishable, both infectible to man; there are other species of this genus causing avian tuberculosis, infecting fish, snakes, turtles and other cold blooded animals. There are a number of acid fast bacilli which are strictly saprophytic but confusable with M. tuberculosis.

Identifying Characteristics:

- (a) Acid fast bacillus, when stained by Ziehl-Neelsen method.
- (b) Pathogenic to guinea pigs, producing tuberculosis in 6 weeks.
- (c) Growth on special media slow, wrinkly and contains acid fast bacilli.

Collection and Transmission of Specimens for Examination: Sputum, exudates, urine, spinal fluid and tissues may be examined for tubercle bacilli. They should be collected under as sterile precautions as feasible (not possible with sputum) and transmitted in suitable container to laboratory. Sputum collection should be so guided as to provide bronchial material rather than the fluids from the mouth or nose.

Microscopical Examination (directly applied to specimen): A presumptive diagnosis can be made by applying an acid fast stain, such as Ziehl-Neelsen carbo-fuchsin to a slide spread of selected (caseous) fragments of the specimen. The red acid fast bacilli will be readily noted in contrast to the blue of the counterstain of all other bacteria cells and debris. Stained spreads may be made from the centrifuged sediment of urine or spinal fluid, using small film of sterile egg albumin on the slide to prevent the sediment being washed off during the staining process.

Concentration Method: If tubercle bacilli are too few to be found by above method they may be concentrated by digesting mucous with sodium hydroxide or antiformin and examining the centrifuged sediment by direct spread, by culture or by guinea pig inoculation.

Sodium Hydroxide Method: Mix equal parts of the specimen and a 3% NaOH solution, shake well, incubate at 37° for 1/2 hour, add HCl to neutrality to litmus, centrifuge and use sediment for test.

MYCOBACTERIUM LEPRAE  
(Leprosy bacillus)

Description: Small slender rods resembling tubercle bacilli, straight, rarely bent or curved, pointed ends; acid fast; tend to be arranged in packets or bundles; cannot be cultivated; not pathogenic to guinea pig.

Habitat: Found in the various lesions of leprosy, except the anaesthetic areas of nerve leprosy; especially demonstrable in nasal and skin lesions.

Identifying Characteristic: 1. Acid fast bacilli occurring in packets.  
2. Not recoverable in culture or guinea pig.

Collection of Specimen: (1) Nasal lesions: As the initial lesion of leprosy is often an ulcer at the junction of bony and cartilaginous septum, swabs or scrapings from this or other nasal lesions are spread onto glass slides.

(2) Skin lesions: With a sterile safety razor blade quickly make a small incision through the thickened area and without removing it depress the upper edge so that a scraping is made of the cut skin from below upward. Prepare slides from this scraped material. The deep, not the surface skin scraping is desired for the spread.

Microscopic Examination: Spreads made as above are fixed stained by Ziehl-Neelsen method and observed for acid fast bacilli. They are more easily decolorized than are tubercle bacilli, though decolorization must not be carried too far. Typical packet bundles of lepra bacilli, or in the skin nodules, lepra bacilli packed in "lepra cells" or in endothelial cells, are conclusive of leprosy.

C.G.S.  
10/7/40.

SPUTUM EXAMINATION FOR TUBERCLE BACILLI  
(Sodium Hydroxide-alum flocculation Method)

Reagents:

1. Digestor: Sodium Hydroxide 40. (4%)  
Potassium Alum 2. (.2%)  
Bromthymol blue .02 (.002%)  
Water to 1000.  
Yellow → Blue  
Note: Range of indicator 6.0 — 7.6  
pH 7.0 = Light bluish green.
2. Acid: Hydrochloric Acid, Concentration 250. (25%)  
Water to 1000. (about 2.5 N)

Test:

- (1) Mix sputum (5 cc.) with 1 to 4 parts of digestor.  
Shake well.  
Incubate at 37°, 30 minutes for culture on animals.  
Incubate at 37° to homogeneous mass for concentration.
- (2) Adjust pH to pH 7 with Acid-digestor adjustment.
- (3) Centrifuge at top speed for 5 minutes.
- (4) Remove supernatant fluids.
- (5) a. Spread on slides — heat fix, or  
b. Culture, or  
c. Inject animals with saline suspension of sediment.

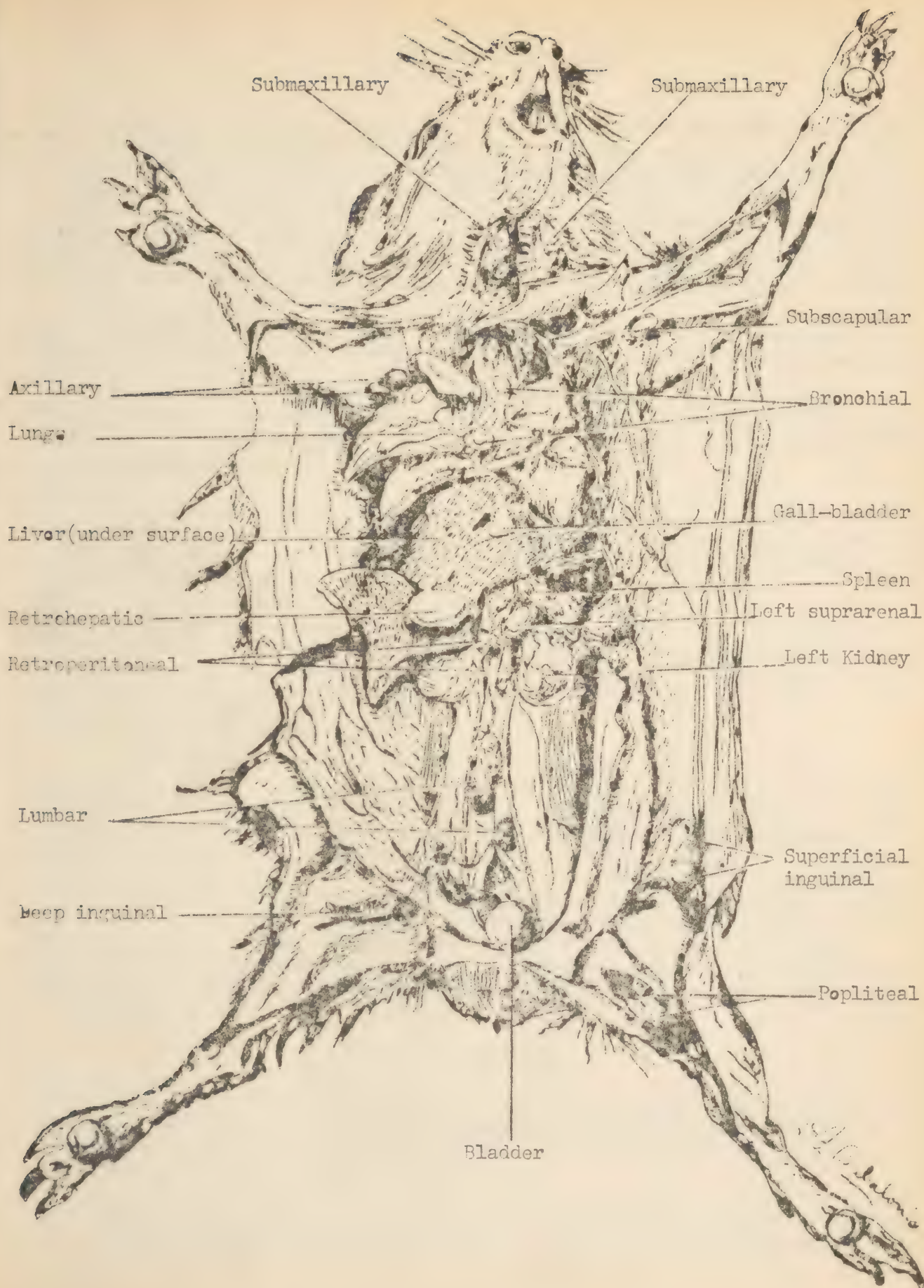
Result: the sodium hydroxide digests the organic matter. Flocculation occurs when acid is added. This flocculation carries into sediment the organisms, including Tubercle bacilli, not killed or dissolved by the alkali.



Animal Inoculation: This is the most certain method of establishing the specific diagnosis of tuberculosis. Centrifuge the NaOH digested sputum or the urine or spinal fluid, suspend the sediment in sterile saline and inject this subcutaneously or intramuscularly into the thigh of a young guinea pig (250 gm.). Autopsy of animal at its death several weeks later, or if it lives at 6 weeks, will reveal generalized tuberculosis, apparent particularly by caseation of glands, spotted liver and large spotted spleen, which may be confirmed by finding acid fast bacilli by direct spread or special culture of those tissues.

Cultural Examination: This is not employed as a routine procedure. Several loopsfull of the sediment in the sodium hydroxide concentrate or tissue fragment from guinea pig tissue, are planted on the surface of tubes of Petroff (or other suitable) medium. Incubate the cultures for two days, then seal - use cotton plugs by dipping them in melted paraffin. Incubate at 37° C. for 6 weeks and examine for colonies of M. tuberculosis.

C.G.S. 10/7/40.



Cadaver of Guinea Pig, Showing Important Organs





## GENERAL CHEMICAL TECHNIC

Chemical analyses, in general, are of two kinds: Qualitative analyses to learn what substances are present in a specimen, or to find out if some particular substance is present, and quantitative analyses to determine the amounts of all or of some of the constituents present. Qualitative analysis tells only if a substance is present; quantitative determination tells how much there is in a particular specimen. The detection of sugar or albumin in the urine is an example of the first; the determination of blood or urine sugar, actually measuring the amount present, is an example of the second.

Quantitative analyses may be made in many ways. The methods most frequently employed are gravimetric, volumetric, colorimetric and gasometric.

In gravimetric analysis the constituent sought is separated from solution as an insoluble compound of known chemical constitution, dried and weighed. From the weight of this compound the weight of the desired constituent is computed.

In volumetric, or titrimetric, analysis the compound sought is not weighed but is determined by measuring the volume of a solution of known concentration which reacts with it. Many procedures in clinical chemistry are based on this principle.

Colorimetric methods are also widely used in the clinical laboratory. The substance to be measured is made to react with other chemical compounds to produce a colored substance. It is essential that the depth of the color produced be proportional to the concentration of the substance sought for in the original specimen.

Similarly in gasometric methods the particular unknown to be measured is made to react to produce a gas. This may be done directly in some cases; in others it is first necessary to combine the unknown with some other compound. In any event it is necessary that the amount of gas produced be in direct relationship to the amount of unknown originally present. The volume of the gas produced is measured under conditions which permit correction of the volume to standard temperature and pressure, or the volume may be held constant and the pressure exerted by the gas be measured on a manometer. From the volume or the pressure, by computation, the amount of unknown is determined. Although not an item of standard equipment, the manometric apparatus of Van Slyke and Neill is used in many of the larger laboratories of the Army.

## CHEMICAL APPARATUS

While it is impossible to consider all of the apparatus used in the chemical laboratory, it is believed advisable to describe the care and use of some articles of equipment and to present certain general methods and technical procedures of wide application.

I. The Chemical Balance.- The balance consists essentially of a horizontal lever with two arms of equal length, and in order to be sensitive and accurate:

The arms must be of equal length; . . .

The point of support must be above the center of gravity;

The fulcrum (knife-edge) and the knife-edges from which the pans are suspended must lie in the same plane and be parallel to one another;

The balance must be level on a vibrationless support.

1. Selection of Proper Types.- Balances which are most suitable for use in the laboratory are of three kinds.

(a) Fine Analytical Balances.- Fine analytical balances are used for gravimetric analyses and for preparing volumetric solutions and other fine weighings. Such a balance should have a capacity from 100 to 200 gm. with a sensitivity of 0.1 mg. when fully loaded. Some of the newer balances such as the "Chainomatic" have devices which obviate the use of fractional weights and greatly expedite weighings.

Precision in weighing can be insured by the use of a standard set of weights, whose accuracy is certified within certain limits by the U. S. Bureau of Standards, or by cheaper sets of ordinary weights on which the corrections have been determined. A standard set of weights may be used to check others, or a set may be calibrated by any one of several methods found in most textbooks on Quantitative Analysis.

(b) Large Balance.- A large balance with a capacity of 2 to 5 kg. and a sensitivity of 0.05 gm. or less.

(c) Trip Scales.- Trip scales to balance tubes for the centrifuge and to weigh materials for the preparation of solutions that do not have to be extremely accurate.

With the introduction of microchemical methods there has been a gradually increasing demand for the microbalance which permits the weighing of minute amounts of material with an accuracy of 0.001 mg.

Since but few laboratories are so equipped and few technicians trained in microgravimetric analysis these methods will not be taken up in this volume.

2. Precautions in Using.- The balance should rest on a firm support which is practically free from mechanical vibration. Direct sunlight should not fall on it as it will cause irregularities and errors in weighing.

When not in use the balance beam should always be raised off the knife-edges or these will be injured by jarring. Always lower the beam slowly and carefully. Never permit the beam to rest on the knife-edges while weights or substances are being added to or removed from the pans.

The beam may be set swinging by dropping the rider upon it or removing it for an instant or by gently fanning one pan with the hand. Never start motion by touching the pan or by suddenly lowering the beam upon the knife-edge.



Make all weighings methodically by trying the weights, one after another in proper order. Before making a weight, see that the balance is properly adjusted. It must be level as shown by the plumb-bob or spirit-level in the case. The pointer must rest at the zero mark with the beam raised. The pointer must swing equal distances to either side of the zero when the beam is set in motion and there is no load on the pans. Adjustment of the swings is made by the balancing screw weights on the ends of the beam. If the balance is equipped with pan arrests, these arrests should be so adjusted that the pointer is on the zero mark when the beam is on its knife-edge and the arrests are in place.

Final adjustments and final weighings should always be made with the balance case closed to prevent errors arising from air currents. When not in use the balance case should be closed and covered with a rubber cover to prevent attack from fumes and gases.

Substances to be weighed should never be placed directly upon the pans, but upon watch glasses, weighing papers, or other containers. The object to be weighed must be at the same temperature as the air within the balance case. Air currents from a hot body and condensation upon the surface of a cold one introduce serious errors. The weight should always be checked carefully; first, by adding up the weights missing from the box (in which every weight should always have its own place); second, by adding up the weights that are on the pan; and last, by adding them up as they are returned to the box.

Never handle weights or place them upon the pans except with the forceps. Always place the substance to be weighed on the left hand pan and the weights on the right hand one, except when the method of double or transposition weighing is used.

3. Methods of Weighing.--(a) Ordinary Method of Direct Weighing.-- Place the object to be weighed on the left pan. If the container is glass or porcelain and has been recently wiped dry, allow it to stand a few minutes in the balance case before weighing it. With the weight forceps place a weight which is judged to be slightly heavier than the object to be weighed on the right pan. Gently lower the beam a little and note which way the pointer swings; if to the left, the weight is too heavy, if to the right, the object is the heavier. Raise the beam and replace the weight with the next heavier one, if it was found to be too light. Continue the trials until a weight is found that is too heavy, always remembering to have the beam raised whenever anything is added to or removed from the pan.

Replace the weight which is too heavy with the next lighter one and try the fractional weights in order in the same manner until the range covered by the rider is reached. Close the balance case and adjust the rider until the pointer swings equal distances to the right and left.

Record the weight from the empty places in the box and check by counting the weights on the pan. Recheck the weights as they are replaced in the box.

In making weighings there is a tendency to make the pointer swing through too great an arc. Because of retardation, which is proportional to



the length of the swing, an error is introduced in long swings which becomes inappreciable in short ones. It is easier to read the end-points of short swings because the pointer is moving more slowly, and there is also less danger of parallax error in reading short swings. A swing of two scale divisions to right and left is ample.

Instead of trying to get two exactly equal swings it is easier to adjust the rider until the swings are nearly the same, and then estimate from the known sensitivity of the balance how much the rider should be moved to make them equal. Place the rider just a little beyond this point and test the swings again. If the balance is adjusted so that the sensitiveness amounts to 5 scale divisions for 1 mg., then a swing that varies less than one-quarter division on the two sides of the center shows that the weight to the nearest 0.1 mg. has been found. This method makes for speed and with substances that give up or take on water on standing, a rapid weight is more accurate than one that is made slowly. This method of weighing is to be preferred for most work to the precise method which follows.

(b) Method of Swings.— In this method, which is very precise, the zero reading of the balance, without any load, must be determined first. Set the beam in motion and record the turning-points, or extreme positions, of the pointer for an uneven number of swings, usually 5, and take the mean of the readings. The first two swings are inaccurate because of the jar in shutting the balance case, and for other reasons, and are disregarded.

For example, suppose the swings are: Left 6.5, right 2.5, left 6.3, right 2.3, left 6.1. The mean of the three left swings would be  $\frac{6.5 + 6.3 + 6.1}{3}$  or 6.3; of the two right swings  $\frac{2.5 + 2.3}{2}$  or 2.4; and the rest point would be  $6.3 - 2.4$  or 3.9 scale divisions to the left. It is customary to give the minus sign to displacements to the left and the plus sign to those to the right, since these signs indicate whether the observed difference is finally to be subtracted from or added to the weight as given by the weights and rider.

It may be simpler to number the divisions from 0 to 20 from left to right and so give the same algebraic sign to all observed readings. In this case, if both balance arms are equal, the zero point would be at 10.

The zero reading may change during the course of the day and should be determined before each weighing. When a series of weighings are to be made, determine the zero point at the beginning and at the end of the series and use the mean.

Next determine the sensitiveness of the balance for the particular load to be weighed by placing the object on the left pan and the weights on the right until equilibrium is established as nearly as possible. Determine the rest point on the scale in the same manner as in making the zero reading. Add or remove 1 mg. by means of the rider and determine the rest point again. The difference between this and the previous point of rest gives the sensitiveness of the balance; that is, the number of scale divisions equal to 1 mg. in weight with the particular load in question.

Assuming that the first point of rest lies at 4.1 scale divisions to the right with a load of 25.723 gm. and the second rest point, with a load of 1 mg. less, or 25.722 gm., at 1.9 scale divisions to the right, then the sensitiveness of the balance will amount to  $4.1 - 1.9$  or 2.2 scale divisions. Since the zero reading was 3.9 scale divisions to the left and the rest point with a load of 25.723 gm. was at 4.1 divisions to the right, it follows that the object was heavier than the weights in the pan by an amount sufficient to displace the rest point from 3.9 to the left to 4.1 to the right, or 8 scale divisions to the right. The weight equal to this amount of displacement can be calculated from the sensitivity as determined above. Since 2.2 scale divisions correspond to 1 mg., then 8 scale divisions would be  $\frac{8.0}{2.2}$  or 3.64 mg. approximately 3.6 mg. The true weight of the object, therefore, would be  $25.723 - 0.0036$  or 25.7194 gm. The weight is only expressed to the fourth decimal place, since most analytical balances will scarcely detect with certainty less than 0.1 mg.

## II. Cleaning Laboratory Glassware.

For ordinary use laboratory glassware can be cleaned in hot soapy water with the aid of the various types of brushes, then rinsed in hot tap water, then in distilled water and allowed to dry.

Where chemical cleanliness is required one of the more elaborate methods of cleansing must be used.

1. Dichromate Cleaning Solution.— Pour 1 liter of commercial concentrated sulfuric acid into 35 cc. of saturated aqueous solution of sodium dichromate, technical, (the sulfuric acid is Item 10360 and the sodium dichromate is Item 14320 in the Medical Department Supply Catalog).

Soak the glassware in this solution for several hours or overnight and then rinse repeatedly in hot tap water until all traces of cleaning solution are removed. Finally rinse in distilled water and allow to dry. In rinsing note whether the water completely wets all of the interior surface and runs off leaving a thin film. If it collects in drops or patches the glassware is not clean and the process must be repeated. It may be advisable to give a preliminary scrubbing with hot soapy water, followed by rinsing in tap water before using the cleaning solution. If used hot the dichromate sulfuric acid solution is more effective.

2. Cleaning with Nitric Acid.— Hot nitric acid is very effective in removing oxidizable organic matter. It must be used with due caution. Immerse the glassware in a beaker of concentrated nitric acid and heat it to boiling under a hood. Pipettes may be placed in an enameled or earthenware tray or in a tall glass specimen jar and the hot nitric acid poured over them. Rinse thoroughly and dry.

3. Cleaning Fermentation Tubes.— These and other glassware difficult to clean by ordinary methods may be treated by moistening the inside with ethyl alcohol. Pour off the excess, leaving not more than 2 cc. in the tube. Add 10 cc. of concentrated nitric acid, place the tubes in a glass or earthenware vessel under a hood and let stand. A vigorous reaction soon occurs with the evolution of much nitrogen dioxide. When the reaction ceases wash with water. Do not close the tube.



4. Trisodium Phosphate Detergent.- A good cleaning agent may be made by dissolving 60 gm. of trisodium phosphate ( $\text{Na}_3\text{PO}_4 \cdot 12 \text{H}_2\text{O}$ ) and 30 gm. of sodium oleate in 500 cc. of water. Soak the glassware in this solution for fifteen to twenty minutes; then scrub it with a stiff brush, rinse and dry. Glassware used in phosphorus determinations should never be cleaned in this solution.

5. Alcoholic Sodium Hydroxide Cleaning Solution.- For removing tarry and greasy residues this solution is very effective. Dissolve 120 gm. of sodium hydroxide in 120 cc. of water, then dilute to 1 liter with 95% ethyl alcohol. The solution may be used hot or cold and must be followed by the usual thorough rinsing in tap and distilled waters.

### III. Volumetric Glassware.

Volumetric flasks, pipettes and burettes are used for accurate measuring of liquids by volume. For ordinary, rough measurement graduated cylinders may be used. The graduate used in pharmacies has no place in the chemical laboratory.

Manufacturers produce several grades of volumetric glassware of varying accuracy. Catalogs of the laboratory supply firms list burettes, pipettes and volumetric flasks in four different grades as a rule. The least accurate is the "student" or educational grade. Next comes the "re-tested" grade which is sufficiently accurate for rough quantitative work. The "precision" grade supposedly fulfills the requirements of the U. S. Bureau of Standards, but each item is not tested and certificates of accuracy are not furnished. This grade is suitable for all but the most exacting work. The highest grade of glassware available is "certified". This means that the individual piece has been tested by the U. S. Bureau of Standards and complies with their specifications. Each piece is etched with the official certification stamp and a certificate is furnished with it.

As furnished by the Medical Department Supply Depots, volumetric glassware is supposed to conform to certain specifications, as a rule corresponding to the "re-tested" grade. For most clinical purposes such as gastric analyses, urine sugar, many blood chemistry determinations, etc., the accuracy is sufficient. In case of doubt, however, the glassware should be calibrated by the methods given in the following pages. This is especially necessary when preparing volumetric solutions for the standardization of other solutions, in preparing all standards for blood chemistry, and in making such solutions as normal sodium hydroxide for use in preparing salvarsan for intravenous injection.

1. Volumetric Flasks.- For general use in the preparation of accurate solutions measuring flasks are a necessity. Convenient sizes are 1000-cc., 500-cc., 250-cc., 100-cc., 50-cc. and 25-cc. More rarely, the 10-cc. size is needed.

The level at which the upper surface of the liquid stands must be in the neck of the flask, and the neck should be narrow to insure accurate readings. Most flasks are calibrated to contain a definite amount and have only



one mark etched on the neck. Some may have two marks, the lower one to contain, the upper to deliver, the specified volume of solution. The calibration mark of the manufacturer may be accepted as correct in the better quality flasks, but for the highest degree of accuracy it may be necessary to check the mark and recalibrate the flasks. This is done as follows:

Cleanse and dry the flask, then weigh it on a balance of appropriate size and sensitivity, taking into consideration the fact that the water to be introduced should be weighed to 1 part per 1000; a 10-cc. flask must be weighed to 0.010 gm., a liter flask to 1 gm. Instead of weighing the flask, it may be counterpoised with shot. Calculate the weight of water necessary to fill the flask to the mark at the proper temperature from Table 1 and place this weight on the balance pan. Fill the flask with distilled water which has been freshly boiled and cooled until balance is attained, making sure that no drops of water adhere to the neck of the flask. Mark the lowest point of the meniscus with a wax pencil sharpened to a chisel edge, then etch in the mark or make a scratch mark with a diamond pencil or sharp file.

TABLE 1

Apparent Weights and Volumes of Water Weighed in Air.  
(For use in calibration of volumetric apparatus)

Temp. °C.	Wt. of 1 cc.	Vol. of 1 gm.	Temp. °C.	Wt. of 1 cc.	Vol. of 1 gm.
15	0.9979	1.0021	23	0.9966	1.0034
16	78	22-	24	64	36
17	77	23	25	61	39
18	75	25	26	59	41
19	73	27	27	56	44
20	72	28	28	54	46
21	70	30	29	51	49
22	68	32	30	48	52

Note: The figures in this table, from Landolt and Borstein's "Tabellen", are based on the weights of water per cc. which must be weighed into a glass vessel under ordinary conditions in order to indicate the mark to which the vessel must be filled with freshly boiled and cooled distilled water so that it will contain the desired volume at 20°C. Corrections for the buoyant effect of air upon vessel and weights are included.

2. Pipettes.—Pipettes are tubes adapted for delivering smaller fixed quantities of liquid than measuring flasks. A pipette for delivering a fixed volume is called a transfer pipette while one for delivering varying quantities is known as a measuring pipette. Pipettes must be narrow at each end and the graduation at the top should be at a sufficient distance to safeguard sucking liquid into the mouth. As with flasks it may be desirable to have two graduations on transfer pipettes, one to mark the point to which the pipette must be filled to deliver the indicated volume and the other to mark the total content.

(a) Calibration of Pipettes for Delivery.- Pipettes are calibrated by weighing the water which they deliver into a weighing bottle which contains a layer of paraffin oil a few mm. thick to prevent evaporation. If the mark is not accurate another mark is then made with a wax pencil and tested. This procedure is repeated until the mark is located correctly. The mark is then etched in. The weight of 1 cc. of water at any temperature from 15 to 30° C. may be found in Table 1.

If an uncalibrated pipette is to be marked, two preliminary marks separated by a definite distance, 50 mm., are made on the stem by a chisel-edged wax pencil, and the water delivered from each is weighed. From the difference, the weight of water contained in each millimeter length of the stem is calculated, and from this the number of mm. from either preliminary mark to the correct mark. The latter is located at the calibrated level on the stem, is tested by weighing the water delivered from it, and finally is etched in.

Example.- The weights of the water delivered at 20°C. by a 10-cc. pipette from two preliminary marks 50 mm. apart are 9.900 and 10.275 gm. respectively. Hence the weight of a column of water between marks is 0.375 gm. or  $\frac{0.375}{50} = 0.0075$  gm. per mm. of column length. The weight of 10 cc. of water at 20°C. is 9.972 gm. or 0.072 gm. more than that delivered from the lower preliminary mark. Hence the correct mark is  $\frac{0.072}{0.0075} = 9.6$  mm. above the lower mark.

(b) Calibration of Pipettes to Contain.- The weight of water required to fill the dry, clean pipette is measured. One may either weigh the pipette empty and full or may fill it with water from a weighing bottle, which is weighed before and after. Other details are the same as for delivery pipettes.

(c) Use of Pipettes.- The Ostwald-Folin type of pipette with a relatively large bulb and short delivery tip is usually calibrated by the manufacturer to deliver the fixed quantity by blowing out the last few drops remaining in the delivery tip. They are marked "TD" - to deliver - and are etched with a single or double broad band near the top. With these pipettes, so marked, the universal practice for all sizes should be to blow out the last few drops.

The volumetric or transfer pipette with the bulb in the middle is also marked "TD". Some, however, and also some Ostwald type pipettes are marked "TC" - to contain. With these marked "to deliver" the practice should be to permit free outflow until the liquid level approaches the lower part of the bulb, then to restrain the outflow with the finger tip, at the same time touching the tip at a slight angle from the vertical, to the wet side wall of the receiving vessel. When as much of the liquid has run out as will do so, remove the finger from the upper end, and hold the pipette tip against the wall of the receiver for a few seconds to make sure that no more of the solution will flow out. Never blow out this type of pipette.

Pipettes marked "to contain" must be rinsed out thoroughly with the diluting solution used in the particular analysis.

Measuring pipettes delivering varying quantities between marks are used more frequently in serology and bacteriology than in chemistry. The outflow should be controlled by the finger and should be slow enough to permit the



walls to drain. Frequently the final graduation to the tip is not accurate and this portion should not be used unless absolutely necessary.

To insure the greatest accuracy in the use of all kinds of pipettes, after filling and before the fluid level is adjusted to the mark, the wet part of the delivery and should be wiped with a clean, absorbent cloth to remove the excess.

3. Burettes.- Burettes differ mainly from measuring pipettes by being open to atmospheric pressure at the top while in use. The outflow at the lower end is started, regulated, and arrested by means of a stopcock or a suitable clamp on an attached rubber tube.

To calibrate a burette of 25 or 50 cc. capacity, fill the clean burette with water to a point slightly above the zero mark. Run out the water slowly until the bottom of the meniscus just touches the mark. Wait a minute for complete drainage of the walls to take place. Adjust the meniscus again to the zero point if necessary. Remove excess water from the tip of the burette. Then run a 2 cc. portion into a weighing bottle containing a little liquid petrolatum, bottle and petrolatum having first been weighed. After delivering the 2 cc. portion, touch the drop adhering to the tip to the surface of the petrolatum carefully so as to remove it. Weigh the bottle and contents. Continue with 2 cc. portions over the entire range of the burette, then take the temperature of the water.

Multiply the weight in grams of each portion of water by the volume of 1 gram at the observed temperature, as taken from Table 1, in order to calculate the actual volume delivered. From the actual volume delivered, make a table of corrections. For ordinary work such as measuring the acidity of gastric contents, the calibration of burettes as made by the manufacturer is sufficiently accurate. For more exact volumetric work the best grade of standardized burette must be used or it must be calibrated in the laboratory.

The glass stopcocks of burettes require especial care. They must be kept clean and lubricated to prevent "freezing" and leakage. Various lubricants are given in this Chapter under the heading "Useful Laboratory Arts and Recipes". A minimum of lubricant should always be used, and the old removed before applying fresh.

Alkali solutions of greater strength than 0.1 N should not be allowed to stand in burettes overnight, otherwise the glass stopcock will "freeze".

#### IV. Filters and Filtration.

The purpose of filtration is to separate a solid from the liquid in which it is suspended, consequently the pores of the paper or the interstices between the matted fibers of other filtering materials must be smaller than the particles which are to be retained. Since precipitates vary greatly with respect to size, proper selection of the paper or other agent is essential.

1. Paper Filters.- The desirable qualities in filter papers for quantitative work are strength, uniform texture, proper porosity and low ash.



For qualitative work strength and proper porosity are required. Select the paper best suited to the particular work to be done. All catalogs of laboratory supply houses give the characteristics of the various filter papers handled by them and in each package of Whatman filter papers is a user's guide indicating the correct paper for each purpose.

An example of improper selection is the use of ordinary paper for filtering trichloroacetic acid precipitated blood serum in calcium determinations, since ordinary papers contain considerable calcium which is dissolved out by the acid. In this case only an acid-washed paper is suitable. On the other hand a blood filtrate for nitrogen determinations should not be filtered through an acid washed paper. In the manufacture of these papers the acid is neutralized with ammonia and some ammonium salts remain in the paper. The blood filtrate dissolves this out, giving a falsely high reading for nitrogen.

The simplest way to fold a circular cut paper is to crease it across one diameter and then, without opening the paper, make a second fold at right angles to the first one. It is important that the paper fit tightly against the walls of the funnel over the entire extent. The paper should be of such size that its upper edge is below the rim of the funnel, especially when precipitates are to be washed.

Although the method just described is the easiest and quickest for folding a paper, considerable time in filtration may be saved by folding the paper so that the liquid passes through it more rapidly. The following method accomplishes this purpose.

1. Fold the paper evenly across one diameter.
2. Open up the paper and make a second fold at right angles to the first, creasing the paper on the same side.
3. Open and turn the paper over, then make a third fold exactly bisecting two of the quarters, creasing on the opposite side from the first two creases.
4. Make a fourth fold at right angles to the last one.

The paper is now divided into eighths, each segment  $45^\circ$  of the circle, with two creases on one side and two on the other.

5. Fold again on the same side as the last, dividing two opposite eighths equally.

6. Make a final fold at right angles to the previous one, and on the same side.

In making the folds be sure all creases pass through the same center point and protect this point with a finger as the creases are made to prevent tearing it.

As the paper is picked up it will tend to shape itself into a cone. Adjust three of the folds, reserving the fourth one to make the paper fit the

funnel exactly. This folding gives alternate triple and single thicknesses with half of the funnel area covered singly. It makes for very rapid filtration and easy washing of precipitates.

## INDICATORS

I. General Considerations.- Indicators, especially those used in acidimetry and alkalimetry are dyestuffs which are of one color in acid solution and another in dilute alkali. The change is due to a re-arrangement within the molecule with a consequent change in the color. It should be remembered that when the completion of a reaction is ascertained by means of an indicator, or by means of any other final color change, it is essential that one solution be added to the other in all cases in the same order, otherwise the results obtained will not correspond exactly.

Solutions should also be of fair concentration and of approximately equivalent strength. Undue dilution causes some indicators to undergo hydrolytic dissociation and this prolongs the end reaction. Dilution in any case tends to prevent a sharp end point. In any titration only a small amount of indicator should be used.

There are many indicators used for various purposes. Those generally useful will be considered in this section, while others for specific determinations will be described in later chapters.

## II. Preparation of Indicator Solutions.

1. Methyl-Orange (Tropaeolin D).- In alkaline solution its color is yellow, but the color changes to red on the addition of a mineral acid. This change of color is not produced by carbonic or other feeble acids. Hence this indicator may be used for the titration of the more powerful mineral acids in the presence of carbonic acid and the feebler organic acids. The presence of much water causes the red color of a faintly acid solution of methyl-orange to become yellow, probably due to hydrolytic dissociation.

The indicator solution generally used is prepared by dissolving 0.1 gm. in 100 cc. of distilled water. One drop of this solution is used for each 20 cc. of solution to be titrated.

A somewhat more sensitive solution, but one in which it requires more experience to detect the color change is prepared by dissolving 0.02 gm. in 100 cc. of hot water, allowing the solution to cool, and filtering out any deposited *m*-sulfonic acid. This more dilute indicator is to be preferred for very exact titrations with very dilute acids and alkalies.

2. Methyl-Red.- This indicator is of value in titrating weak organic bases and ammonia. The aqueous solution is orange colored, but if a few drops are added to 50 to 100 cc. of water, the latter is colored a pale yellow. The addition of a drop of 0.1 N HCl at once turns the liquid a violet-red without passing through any intermediate shade and by the addition of a drop of ammonia the solution becomes nearly colorless again. Methyl-red is not very sensitive to carbonic acid, but more so than methyl-orange, so that it is less



suitable for the titration of carbonates. The chief advantage of this indicator lies in its sharp color change from a very pale yellow to a violet-red even in titrating ammonia.

To prepare the indicator solution, dissolve 0.02 gm. of the free acid in 100 cc. of hot water, allow to cool and filter. Add 2 to 3 drops of this solution to every 100 cc. of the solution to be titrated.

3. Phenolphthalein.- The solution of this indicator in alcohol is colorless. When a few drops are added to the solution of an alkali, the liquid assumes an intense red color; this color is readily destroyed by the addition of an excess of mineral or organic acid. Phenolphthalein is suitable for the titration of organic and inorganic acids and of strong bases, but it cannot be used in the presence of carbonic acid, or of ammonium salts; it is therefore not suitable for titrating ammonia by an acid.

The presence of free carbon dioxide in ordinary distilled water causes a slight error, which should be allowed for, particularly if 0.1 N or 0.01 N acid solutions are used.

Concentrated solutions of alkaline hydroxides do not give a red color with this indicator; such solutions should therefore be diluted with water before titration.

The stock solution is prepared by dissolving 1 gm. of pure phenolphthalein in 60 cc. of absolute alcohol, adding 40 cc. of water, and then filtering if necessary. From this a 0.5 per cent solution in 50 per cent alcohol may be made.

The 1 per cent and 0.5 per cent solutions are used in the proportion of 1 drop to 10 cc. or more of solution. A more sensitive indicator is prepared by diluting the one per cent stock solution ten times with 50 per cent alcohol. One to four drops of this 0.1 per cent indicator is used for each 100 cc. of solution. The stronger indicator solutions are used in titrating gastric contents for total acidity while the weaker is used for the exact titration of strong acids and bases.

## VOLUMETRIC SOLUTIONS

### I. Definitions.

1. Molar Solution.- A molar solution of any chemical compound is a solution of such concentration that 1 liter contains 1 gram molecule of the compound. The molecular weight in grams of the compound is dissolved in water or other solvent and made up to 1 liter. For example, the molecular weight of sodium chloride is 58.454; a molar solution would contain 58.454 gm. of sodium chloride in 1 liter of solution; the molecular weight of sulfuric acid is 98.076, so a molar solution would contain that weight in grams per liter of solution.

2. Normal Solution.- By a normal solution of any compound is meant one which contains one gram-equivalent of the active reagent in 1 liter of solution. By gram-equivalent is meant the amount of substance equivalent to one gram-atom.



(1.008 gm.) of hydrogen. A milli-equivalent is the thousandth part of a gram-equivalent. Stated differently a normal solution is one which contains 1 gram-atom (1.008 gm.) of reacting hydrogen per liter of solution, or which can quantitatively replace or react, directly or indirectly, with an equal volume of such a solution.

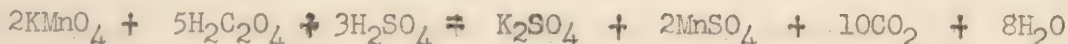
Of the various types of normal volumetric solutions the ones most commonly used in clinical laboratories fall into the following classes:

(a) Normal Acid Solutions.- In acid-alkali titration, a normal acid solution contains per liter the amount of acid that has 1 gm. atom of hydrogen replaceable by alkali at the pH used as the end-point in titration; e.g., 1 molecule of HCl contains 1 atom of replaceable hydrogen, therefore a liter of a normal solution of this acid contains 1 gm. molecule or 36.465 gm. of HCl. One molecule of sulfuric acid,  $H_2SO_4$ , contains, however, for titration to ordinary end-points, 2 atoms of replaceable hydrogen; therefore a liter of normal solution of this acid contains  $\frac{1}{2}$  gm. molecule, or  $0.5 \times 98.076$  or 49.038 gm. of  $H_2SO_4$ .

(b) Normal Alkali Solutions.- A normal alkali solution is one which will neutralize, volume for volume, a normal acid solution. A normal solution of sodium hydroxide, NaOH, is molar, but one of barium hydroxide,  $Ba(OH)_2$ , is half molar. Such solutions are normal in hydroxyl; that is they contain one gram-equivalent of OH in each liter of solution.

(c) Normal Reducing Solutions.- A normal reducing solution is one which contains in 1 liter 1 gm. atom of oxidizable hydrogen or its equivalent in other reducing substances. Oxalic acid,  $H_2C_2O_4$ , has two hydrogen atoms, both of which are titratable with alkali, and both of which are oxidizable by permanganate. Hence a normal solution of oxalic acid, whether for acidimetry or for oxidation by permanganate is half molar.

(d) Normal Oxidizing Solutions.- A normal oxidizing solution is one a liter of which will oxidize 1 gm. atom of hydrogen, or its equivalent of other reducing substances. In the reaction



each molecule of permanganate oxidizes 5 of the hydrogen atoms of the oxalic acid. Hence a normal solution of permanganate is only one-fifth molar.

3. Acidimetry and Alkalimetry.- Acidimetry and alkalimetry cover the analysis of acids and bases. In order to determine the amount of acid present, an alkaline solution of known strength is required; and conversely in the analysis of a base an acid solution is required. In both cases the "end point" of the reaction is determined with the help of a suitable indicator.

4. Oxidation and Reduction.- In oxidation and reduction methods the substance analyzed is either oxidized or reduced by means of the solution with which the titration is made. When hydrogen is oxidized by oxygen it is changed from the neutral condition to that of a positive valence, or polarity, of one, and the oxygen is reduced from the neutral condition to a negative valence, or

polarity, of two. In this, and all other cases, the equivalent weight of the element used in an oxidation-reduction reaction is the atomic weight divided by the change in polarity. When the atom of any complex molecule is subjected to a change in polarity, oxidized or reduced, the equivalent weight of the molecule is the gram-molecular weight divided by the change in polarity of the oxidized or reduced element. If more than one atom of the reactive element is present in the molecule, the molecular weight is divided by the total change in polarity, i. e., by the change in polarity multiplied by the number of atoms undergoing such change.

As an example, potassium dichromate is often used as an oxidizing agent. In it each chromium atom has a polarity of +6 and by reduction two trivalent chromic ions are formed. There is a loss in polarity of 3 charges for each chromium atom and a normal solution of potassium dichromate,  $K_2Cr_2O_7$ , will contain one-sixth of a mole.

In a like manner, the equivalent weight of a reducing agent is determined by the gain in polarity which the oxidizing element experiences. Ferrous salts are oxidized to ferric salts and the iron is changed from +2 to +3 in polarity.



5. Precipitation and Substitution Reactions. In precipitation and substitution reactions accurate determinations are made by precipitating or substituting a final product by means of a normal solution. The classical example is the extensively used method for testing silver alloys and this depends upon the precipitation of silver chloride from nitric acid, common salt solution of known concentration being used as the precipitant.

## II. Preparation of Standard Solutions.

### 1. Normal Hydrochloric Acid.- $HCl = 36.465$ .

(a) Constant Boiling Mixture Method. Hulett and Bonner.- This method, which is capable of accuracy to 1 part in 10,000, depends on the fact that when hydrochloric acid solution is distilled, the concentration of acid in the undistilled portion approaches a constant concentration of 20.22 per cent of  $HCl$  by weight after distillation at 760 mm. of mercury pressure. If boiling is continued water and hydrochloric acid distil off in constant proportions identical with those in the residual undistilled fraction.

To concentrated hydrochloric acid, specific gravity 1.2, add an equal volume of water. Bring the solution to a density of 1.096 at 25°C. by the addition of more water or acid. Distil away three-fourths of the mixture at a rate of 3 or 4 cc. per minute. Save this distillate as the starting point for a second lot, adjusting it to a specific gravity of 1.096 at 25°C. The remaining one-fourth has, within 1 part in 10,000, the composition given in Table 2. Of this quarter, all but the last 50 to 60 cc. is distilled, and the distillate used to prepare standard solutions. The barometer is recorded at the time of distillation. In glass-stoppered bottles such solutions keep indefinitely.



A 0.1 N standard solution is prepared by diluting the proper weight, not volume, of the acid, estimated from Table 2, to volume with water. Exactly 16.4 cc., approximately 18 gm., of the acid are measured into a 50-cc. flask. More acid is added or withdrawn by means of a medicine dropper with a fine drawn out tip, until the exact weight as given in the table is obtained. The acid is then diluted with water, rinsed into a 1 liter volumetric flask which has been calibrated, and diluted to volume. Further standardization is unnecessary. In fact, acid prepared in this manner can be relied upon for the standardization of alkali and other reagents. In this and all other volumetric solutions, final adjustment of the volume in the flask must be made at 20°C. since the flasks are calibrated at that temperature. Use of distilled water at 20°C. facilitates this adjustment.

TABLE 2

## Hulett and Bonner's Constant Boiling Hydrochloric Acid

(Values for pressures 730 to 770 mm. corrected by Foulk and Hollingsworth.)

(Values for pressures 620 to 660 mm. added by Bonner and Branting.)

Barometric pressure at time of distillation. mm. Hg.	HCl concentra- tion by weight.. Per cent.	Solution required to make 1 liter of 0.1 N HCl Grams.
620	20.560	17.719
630	20.532	17.743
640	20.504	17.767
650	20.471	17.800
660	20.438	17.824
730	20.293	17.956
740	20.269	17.977
750	20.245	17.998
760	20.221	18.019
770	20.197	18.041

(b) Standardization of Other Acids by Comparison with Standard HCl.—Hydrochloric acid standards prepared by weight from Hulett and Bonner acid, as described above, may be used to standardize any other acid solution by the following procedure.

With a pipette measure 20 or 25 cc. of standard hydrochloric acid into a flask and titrate from a burette with alkali of approximately the same normality. With the same pipette, the same volume of the other acid is measured into a flask and titrated, using the same quantity of the same indicator, with the same alkali from the same burette, which is filled exactly to the zero point at the beginning of each titration.



The factor of the unknown acid is calculated as

$$\text{factor} = \frac{\text{Cc. of standard HCl}}{\text{Cc. of other acid}}$$

This procedure eliminates errors of calibration of different pieces of apparatus and those from deterioration or inaccurate standardization of alkali.

(c) By Standardization.- Normal hydrochloric acid contains in 1000 cc., exactly 36.465 gm. of HCl. Dilute pure, concentrated hydrochloric acid with 11 volumes of water. In this way a solution is obtained that is slightly more than normal in strength. To obtain an exactly normal solution, it may be titrated against a weighed amount of chemically-pure sodium carbonate, and from the results obtained the volume of water to be added computed, or it may be standardized gravimetrically with calcite (Iceland spar) as follows:

To a weighed crystal of Iceland spar in a beaker, add a known volume, 50 to 100 cc., of the acid to be standardized. Cover the beaker with a watch glass and set aside until the action of the acid on the crystal has completely ceased. Remove the crystal from the solution, wash in distilled water, dry and reweigh. The loss of weight is due to the solution of the carbonate under the influence of the acid.

Then  $\frac{20 \times \text{gm. of Iceland spar dissolved}}{\text{cc. of acid used}} = \text{factor for 1 N acid.}$

If the acid is made up somewhat stronger than 1 N, and a given volume of it is diluted to factor times that volume, the diluted acid will have a factor exactly 1.0000.

2. Normal Sulfuric Acid.-  $\text{H}_2\text{SO}_4 = 98.076$ . This solution is half-molar, and therefore contains 49.038 gm. in 1000 cc. Add slowly, with constant stirring, 30 cc. of sulfuric acid to 1020 cc. of distilled water and allow to cool to  $25^\circ\text{C}$ . Ascertain its exact strength by titration against freshly standardized normal sodium hydroxide, using methyl orange indicator, and adjust to exact normality or to a known strength of approximate normality.

The strength may also be ascertained and adjusted after titrating an accurately weighed amount of reagent anhydrous sodium carbonate. It may also be standardized gravimetrically in the same manner as hydrochloric acid.

3. Normal Oxalic Acid.-  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O} = 126.068$ .- This solution is a half-molar one. Dissolve 64.5 gm. of reagent oxalic acid in sufficient distilled water to measure 1000 cc. Ascertain its exact strength by titration against freshly standardized normal sodium hydroxide, using phenolphthalein indicator, and adjust to exact normality or to a known strength of approximate normality.

4. Normal Sodium Hydroxide -  $\text{NaOH} = 40.005$ .- Dissolve 100 to 500 gm. of the purest sodium hydroxide obtainable in an equal amount of water in a Pyrex flask. The solution becomes very hot. Cool to room temperature and transfer to a paraffin lined bottle or cylinder, stoppered with a paraffined stopper.

In such a concentrated solution carbonate is almost insoluble and will settle out in a few days. The clear concentrated solution contains about 70 gm. of NaOH to the 100 cc.

Prepare the normal solution by diluting 60 cc. of the clear concentrated solution to 1 liter with distilled water. Standardize the hydroxide solution as follows: Place 25 or 50 cc. of standardized normal hydrochloric acid in a flask, add a drop or two of methyl orange or phenolphthalein indicator and run in the alkali solution from a burette until the end point is reached. It is best to run the alkali into the acid since the alkali has less opportunity to absorb  $\text{CO}_2$  from the air if in a burette and also because the change in the indicator is more definite.

Calculate the normality factor of the hydroxide by the formula factor =  $\frac{\text{cc. of 1 N HCl}}{\text{cc. of NaOH}}$ . If the normality factor is greater than 1, indicating that the solution is too strong, the concentration of that remaining can be reduced to exactly 1 N by diluting the total volume to the figure obtained by multiplying the cubic centimeters remaining by the normality factor. For example, the normality factor is found to be 1.020 and there are 950 cc. of hydroxide solution left from the titration. Multiplying 950 by 1.020 gives 969, the volume to which the hydroxide should be diluted. This is done by adding 19 cc. of water. Again titrate and calculate the normality factor.

Note.— Solutions of alkali hydroxides absorb carbon dioxide when exposed to the atmosphere. They should therefore be preserved in Pyrex bottles with well-fitting rubber stoppers, provided with tubes filled with a mixture of sodium hydroxide and lime (soda-lime tubes) so that air in entering the container must pass through these tubes which will absorb the carbon dioxide.

5. Normal Sodium Carbonate.—  $\text{Na}_2\text{CO}_3 = 106.004$ .— This may be prepared directly from anhydrous sodium carbonate of the best quality (American Chemical Society Specifications or Standard of Murray) by weighing out slightly more than one-half the molecular weight, dissolving it in distilled water and making up the volume to 1 liter. Ordinarily 53.5 gm. will give a solution but slightly stronger than normal.

Standardize by titrating 50 cc. of the carbonate solution with 1N HCl, using methyl orange as an indicator.





## EXAMINATION OF THE URINE

## COLLECTION AND PRESERVATION OF SPECIMENS

## I. Samples.

1. Single samples. These are used as a matter of convenience but only for qualitative examination. Abnormal findings in such a specimen should be checked by the examination of a twenty-four-hour specimen. Of the single samples, one passed three hours after a meal is best. The single sample voided on arising is least likely to yield pathological findings, yet it may be at times the most desirable for checking the presence of pus and mucus.

2. Day and night samples. In certain conditions and for some tests it may be desirable to collect the day and night urines separately. Usually all urine voided from 8 A. M. to 8 P. M. is collected as the day sample and that from 8 P. M. to 8 A. M. as the night sample.

3. Twenty-four-hour sample. This sample is preferred for routine examination and is absolutely essential for accurate study of kidney conditions. No quantitative test should be done except on a representative portion of a twenty-four-hour sample. For convenience the collection is usually started in the morning, at 7 A. M. The patient voids and this voiding is discarded. All urine passed subsequently is saved. At 7 A. M. the following morning the patient voids and this urine is added to the sample. The volume is noted, the total specimen is well mixed and 120 to 240 cc. sent to the laboratory for analysis.

## II. Preservatives.

1. Cold.- If a refrigerator is available, samples may be kept in it until examined. Samples for pregnancy tests must be so preserved. Avoid freezing.

## 2. Chemical preservatives.

(a) Toluene is the best chemical preservative. Sufficient should be added to form a thin layer on the surface.

(b) Camphor. A small piece sufficient to give a saturated solution is used.

(c) Formaldehyde. Two drops of the ordinary commercial solution to an ounce of urine are sufficient. If in excess it interferes with albumin, sugar and indican tests. It is most satisfactory for the preservation of formed elements.

## PHYSICAL EXAMINATION

I. Color.- Various shades of yellow and amber are normal, the darker shades being associated with increased concentration and high specific gravities, the lighter with dilution and low specific gravities. Increased amounts of the normal urinary pigments - urochrome, urobilin and uroerythrin -, the presence of abnormal pigments, and the renal elimination of drugs may produce a variety of colors.

Color is usually recorded as straw, yellow, amber, etc., indicating the shade as light, medium or dark; i.e., light straw, dark amber, etc.

II. Appearance.- Normal, freshly-passed urine is clear and sparkling. On standing a cloud may appear consisting of mucus with epithelial and pus cells entangled in it. Concentrated urines, on cooling, may develop a white, pink or reddish sediment. Pus or blood if present produce a cloudy or smoky appearance. During the change to alkalinity in older specimens phosphates may separate out. Marked cloudiness in a freshly-passed specimen is significant of a pathological condition. Appearance is usually described as clear, slightly cloudy, very cloudy; to describe the sediment: slight, moderate or heavy; and its color: white, pink, reddish, etc.

III. Reaction.- Determine whether the reaction is acid, alkaline or neutral by the use of blue and red litmus paper. Freshly voided normal urine is usually acid, averaging about pH 6.0. Properly preserved twenty-four-hour samples are also slightly acid. The diet is the most important factor in modifying the reaction of the urine.

IV. Specific Gravity.- This is most conveniently estimated with the urinometer, a special type of hydrometer. Each urinometer is calibrated to give readings at a definite temperature, usually 25°C. (77°F.); this calibration is marked on the spindle. If the temperature of the urine is above that at which the urinometer is calibrated, a correction of 0.001 must be added for each 3°C. above the standard temperature and similarly deducted for each 3°C. below this calibration temperature. For example, if the urinometer, calibrated at 25°C., reads 1.018 in a urine at a temperature of 31°C., then 0.002 must be added, giving the corrected specific gravity of 1.020. Normally the specific gravity varies between 1.015 and 1.030; pathologically it may range from 1.000 to 1.060.

V. Quantity.- Normally 1200 to 1500 cc. are passed in twenty-four hours. In diabetes mellitus and diabetes insipidus the quantity is much increased. In water loss due to diarrhoea, excessive vomiting or profuse sweating the amount is decreased. Normally the day volume exceeds the night volume, being three to four times as much.

VI. Total solids.- The amount of solids excreted in the urine may be roughly calculated by means of Long's coefficient, 2.6. The last two figures of the specific gravity taken at 25°C. multiplied by this coefficient gives the number of grams of total solids in 1000 cc. of urine.



## ROUTINE CHEMICAL EXAMINATION

I. Albumin.- All normal urines contain some albumin, but the amount is so small it escapes detection by the methods generally used. The principles employed are either coagulation by heat or precipitation by chemical agents. No test is absolutely satisfactory due to interference of other substances precipitated with the albumin. Mucin is a common source of error. When present it can be removed by adding acetic acid and filtering. All tests for albumin require that the urine be absolutely clear; cloudy samples should be cleared by filtration or centrifugalization, otherwise reactions due to small amounts of albumin will be masked in the general turbidity.

## A. Heat and Acetic acid Test.

1. Reagent.- Acetic acid, 5 per cent

2. Procedure.- Fill a test tube two-thirds full of clear urine. Heat the upper portion gently to boiling in an open flame. Precipitates forming at this point may be due to albumin or phosphates. Add 3 to 5 drops of 5% acetic acid solution, a drop at a time.

3. Results.- If the precipitate dissolves, it was due to phosphates. If due to albumin, the precipitate will become heavier and more flocculent. Compare the cloudiness produced with the unheated urine in the lower portion of the tube.

## B. Nitric Acid and Magnesium Sulfate Ring Test. (Roberts').-

1. Reagent.- To 5 volumes of saturated aqueous solution of magnesium sulfate, U.S.P., add 1 volume of concentrated nitric acid.

2. Procedure.- Place a few cc. of reagent in a test tube, tilt the tube, and introduce the urine sample with a pipette, allowing it to flow gently down the side of the tube so as to underlay the reagent without mixing. If albumin is present a fluffy, white ring of precipitated albumin forms at the juncture line.

Another method uses a pipette made of glass tubing with an inside diameter of about 5 mm. Place a few cc. of Roberts' reagent in a test tube. With the pipette take up a small column of urine, about 1 cm. long, wipe excess urine from the outside, then place it in the test tube carefully, holding the finger firmly over the upper end until the other end touches the bottom of the tube. Release the finger pressure gradually, allowing the reagent to rise in the pipette, forming a clear, distinct layer with the urine. After standing for a minute or two, read against a dark background.

3. Results.- A white ring at the junction of the liquids indicates albumin, the thickness and density of the ring showing the amount. No confusing colored rings due to indican, iodides, bile pigments or the oxidation products of organic constituents are formed as is frequently the case when nitric acid alone (Heller's Test) is used. A white ring or cloudiness may form above the contact zone, due to urates or mucus, but such rings are less



sharp, broader, and lie above the albumin ring when both are present.

### C. Sulfosalicylic Acid Test (Exton).-

1. Reagent.- Dissolve 200 gm. of crystalline sodium sulfate ( $\text{Na}_2\text{SO}_4 \cdot 10 \text{H}_2\text{O}$ ) in 800 cc. of water with the aid of heat. Cool to  $35^\circ\text{C}$ . and add 50 gm. of sulfosalicylic acid. Dissolve and dilute to 1 liter.

2. Procedure.- Mix equal volumes of clear urine and reagent in a test tube. Warm the mixture gently; do not boil.

3. Results.- If cloudiness does not develop in the cold, albumin is absent. If cloudiness appears and persists or increases on gentle heating, albumin is present.

### D. Osgood-Haskins' Test.

1. Reagents.- (a) Acetic acid, 50% solution.

(b) Sodium chloride, saturated aqueous solution (30%).

2. Procedure.- To 5 volumes of urine in a test tube, add 1 volume of the 50% acetic acid, followed by 3 volumes of the saturated sodium chloride solution. Heat the mixture gradually to boiling.

3. Results.- A precipitate appearing upon the addition of the acid indicates bile salts, urates or resin acids, etc., whereas a precipitate appearing after the addition of the salt solution suggests Bence-Jones protein, or globulin in excess of 0.38 gm. per liter. As the temperature is raised, the precipitate of Bence-Jones protein, if present, will go back into solution; if albumin or globulin are present a precipitate will form. This test has the advantage of indicating the presence of Bence-Jones protein as well as albumin and globulin.

## II. Glucose.

### A. Benedict's Test.

1. Reagent.- (a) Benedict's Qualitative.

Cupric sulfate crystals ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) U.S.P. 17.3 gm.

Sodium carbonate, monohydrated U.S.P. 117.0 gm.

(or Sod. carbonate, anhydrous U.S.P. 100 gm.)

Sodium citrate, U.S.P. 173.0 gm.

Dissolve the copper sulfate in about 100 cc. of water.

Dissolve the carbonate and citrate in 700 cc. of water, with the aid of heat, if necessary. Cool to room temperature and pour in the copper solution slowly with constant stirring. When completely mixed make up the volume to 1000 cc.

2. Procedure.- Place 5 cc. of reagent in a test tube. Add 8 drops (0.5 cc.), not more, of urine. Boil vigorously over an open flame for 1 to 2 minutes, then allow to cool spontaneously. Do not hasten cooling by immersion in cold water. If a large number of tests are to be run, the tubes may be placed in a boiling water bath, or a beaker of boiling water, and heated for 5 minutes, then allowed to cool.

3. Results.- In the presence of glucose the entire solution will be filled with a bulky, colloidal precipitate which may be greenish-yellow, yellow or red in color, depending on the amount of glucose present. In the presence of over 0.2 - 0.3% of glucose the precipitate will form quickly. If no glucose is present the solution will remain perfectly clear or will show a faint turbidity due to precipitated urates.

### III. Indican.

A. Obermayer's Test.- This test depends on the decomposition of indican and the subsequent oxidation of the liberated indoxyl to indigo blue, at times to indigo red.

1. Reagents.- (a) Obermayer's reagent.- Add 2 gm. of ferric chloride to 1000 cc. of concentrated hydrochloric acid (sp. gr. 1.19 or 23.5° Baume').

(b) Chloroform.

2. Procedure.- To 5 cc. of urine in a test tube add an equal amount of reagent and 1-2 cc. of chloroform. Mix by inverting 10 times. Allow the chloroform to settle and examine its color.

3. Results.- A pale blue to deep blue to violet color indicates the presence of indican, the intensity of the color being proportional to its concentration. If the oxidation is slow, a red color due to the formation of indigo red may appear. Iodides may give a red-violet color due to the liberation of iodine. The addition of a few drops of a concentrated solution or a small crystal of sodium thiosulfate will discharge this color. Thymol may produce a violet color; the thiosulfate will destroy this also. Bile pigments interfere with the test and must be removed by adding one-fifth volume of 10 per cent calcium or barium chloride solution and filtering.

Urotropin (hexamethylene tetramine) and formaldehyde prevent the appearance of the indigo blue even when indican is present in large amounts.

Report the test as negative, slight excess or large excess. Normal urines may give a faint blue.

### IV. Acetone.

A. Sodium Nitroprusside Test. (Lange's).

1. Reagents.- (a) Acetic acid, glacial (99%).

(b) Ammonium hydroxide 28% (stronger ammonia water).



(c) Sodium nitroprusside, freshly prepared saturated solution. Dissolve several crystals in 1-2 cc. of water by gentle heat, having a slight excess of undissolved crystals remaining.

2. Procedure.- Place 5 cc. of filtered urine in a test tube, add 0.5 cc. of glacial acetic acid and 0.5 cc. of the freshly prepared sodium nitroprusside solution and mix. Tilt the tube and carefully overlay the mixture with 1-2 cc. of strong ammonia water.

3. Results.- A purple or purplish-red ring forms at the contact zone in a few minutes if acetone is present. The ring tends to be more purple or violet in low concentrations, more red-purple in high. Amorphous urates may give a brown or orange ring if present in large amount.

B. Rantzman Modification.- Aqueous solutions of sodium nitroprusside decompose rapidly and hence must be freshly prepared. This modification gives a reagent which keeps fairly well.

1. Reagent.- Dissolve 37.5 gm. ammonium nitrate crystals and 2.5 gm. sodium nitroprusside in distilled water and make up to 100 cc. In a brown glass-stoppered bottle this reagent will keep for 2 months.

2. Procedure.- To 3 cc. of urine in a test tube add 1 cc. of the reagent. Mix and overlay with strong ammonia water.

3. Result.- If acetone is present a sharply defined purple or Burgundy red ring appears at the contact zone. The smaller the amount of acetone present, the longer it takes the ring to appear.

C. Ross Modification of Rothera's Test.

1. Reagent.- Mix one part of powdered sodium nitroprusside and 100 parts of powdered ammonium sulfate.

2. Procedure.- Place 1 gm. of the dry powdered reagent in a test tube and add 5 cc. of clear urine. Mix until the powder is dissolved, then overlay with strong ammonia water.

3. Result.- A red-purple "permanganate" color indicates the presence of acetone.

V. Aceto-acetic acid (Diacetic acid).

A. Ferric chloride Test (Gerhardt).

1. Reagent.- (a) Ferric chloride, 10% aqueous solution.

2. Procedure.- To 5 cc. of urine in a test tube, add the ferric chloride reagent drop by drop until no more phosphates precipitate. Filter and add more ferric chloride solution.

3. Result.- If aceto-acetic acid is present a Bordeaux red color



develops. A similar color is produced by phenols, coal tar antipyretics, bicarbonates, salicylates, etc.

## VI. Bile Pigments.

### A. Rosenbach's Modification of Gmelin's Test.

1. Reagent.- (a) Nitric acid, concentrated, containing nitrous acid.

This is concentrated nitric acid which is slightly yellow, due to the presence of nitrous acid. Colorless nitric acid will become yellow by exposing it to sunlight in a plain glass container for several days. To hasten this change, heat colorless acid in a beaker with a small sliver of pine wood (piece of match stick) until fumes appear.

2. Procedure.- Filter 10-20 cc. of urine, acidified with 1 or 2 drops of dilute hydrochloric acid through a small, heavy filter paper. Introduce 1 drop of the nitric acid into the apex of the paper, then unfold it.

3. Result.- A play of colors appears in the order of green, blue, violet, red and reddish-yellow, the last nearest the center of the paper.

### B. Hammarsten's Test for Bilirubin.

1. Reagents.-

(a) Barium chloride, 10% aqueous solution.

(b) Ethyl alcohol, absolute.

(c) Nitric acid, 1:4. Dilute one part of concentrated acid with 3 parts of water.

(d) Hydrochloric acid, 1:4. Dilute one part of concentrated acid with 3 parts of water.

(e) Stock Reagent. Mix 1 part of the dilute nitric acid and 19 parts of the dilute hydrochloric acid.

(f) Test Reagent. To 1 part of Stock reagent, add 4 parts of the absolute ethyl alcohol.

2. Methods.

(a) With whole urine.- To 2 cc. of test reagent in a test tube, add a few drops of urine.

(b) With urinary precipitate.- To 5 cc. of acid urine (acidify if necessary) add 5 cc. of 10% barium chloride solution. Mix well and centrifugalize. Decant and discard the supernatant. Mix the precipitate with 2 cc. of the test reagent and centrifugalize.

3. Result.- A green color is produced if bile pigments were present. This test is sensitive to 1 part of bile pigment in 1,000,000 parts of urine.

### C. Huppert-Nakayama Test.

1. Reagents.- (a) Barium chloride, 5% solution.

(b) Nakayama reagent: Dissolve 0.4 gm. ferric chloride in a mixture of 99 cc. of 95% ethyl alcohol and 1 cc. of concentrated hydrochloric acid.

(c) Nitric acid, concentrated.

2. Procedure.- To 5 cc. of urine add 5 cc. of the barium chloride solution. Mix thoroughly and centrifugalize. Pour off the supernatant fluid. To the sediment add 2 cc. of the Nakayama reagent, mix and bring to a boil.

3. Result.- A brilliant deep green color develops if bilirubin is present. On adding a few drops of nitric acid the color changes to violet or red.

## VII. Urobilinogen.

Bilirubin excreted into the intestine in the bile is decomposed with the production first of the colorless compound urobilinogen, and then urobilin. Normally these compounds are partially absorbed from the intestine, carried to the liver and reconverted into bilirubin. Some part of the urobilinogen normally finds its way into the general circulation and is excreted in the urine. In disturbances of liver function and in certain toxemias, infectious diseases, and hemolytic conditions larger amounts may get into the general circulation and appear in the urine.

### A. Ehrlich Aldehyde Test.- (Modification of Wallace and Diamond).

This test is roughly quantitative.

1. Reagent.- Dissolve 2 gm. of paradimethylaminobenzaldehyde in 100 cc. of 20 per cent (by volume) hydrochloric acid.

2. Procedure.- To 10 cc. of bile free, undiluted urine at room temperature, or warmed to 21 - 22°C., add 1 cc. of the reagent; allow to stand for three minutes. If a deep cherry red color appears, proceed with the test using 10 cc. portions of dilutions of the urine. Make 1:10, 1:20, 1:50, 1:100 and 1:200 dilutions with tap water at room temperature. Add to the 10 cc. portions, 1 cc. of reagent, let stand 3 to 5 minutes, not longer, and read.

3. Results.- Express the result in terms of the highest dilution giving a faint but definite pink or cherry color; i.e. "Positive in 1:20 dilution". Normally this is at the 1:20 dilution. Any greater dilution yielding a definite pink color indicates a pathological amount of urobilinogen. A daily estimation showing positive in greater and greater dilution is especially significant.



### VIII. Blood.

The detection of traces of blood requires microscopic examination of the urinary sediment for red blood cells and chemical examination for hemoglobin. See "Microscopic Examination of Urine" and the chapter on "Examination of Stomach Contents" for the methods.

#### MICROSCOPIC EXAMINATION OF URINARY SEDIMENTS

Routine examination of the urine should include a careful microscopic examination of the urinary sediment in all cases.

I. Preparation of Specimen.- Urines should be examined within a few hours after voiding, unless kept at a low temperature or preserved with a chemical. Alkaline specimens should be examined as soon as possible. If large amounts of suspended amorphous phosphates in an alkaline urine obscure the field, the specimen should be cleared with dilute acetic acid and re-examined. Heavy urate sediments in an acid urine may be dissolved by gently warming the specimen.

The sediment for examination is usually secured by centrifugalizing a 15 cc. portion of the urine at a relatively low speed, - 1000 to 2000 r.p.m. for 5 minutes. The supernatant urine is poured off as completely as possible and the sediment mixed with the urine remaining by tapping the end of the tube with the finger. A drop of the mixed sediment is removed to a clean glass slide by means of a pipette, or a drop may be poured directly on to the slide from the centrifuge tube.

If a centrifuge is not available, sedimentation in long, conical urine test glasses may be done. This is not as satisfactory as centrifugalization because of the longer time required and because the various constituents, due to differences in specific gravity, settle out at different rates, giving a less homogeneous sediment. A drop of sediment collected in this manner must be transferred to the slide by means of a pipette.

II. Examination of the Drop.- Examination is usually made with a low-power objective (16 mm.) and a moderately high-power eye piece (10x). The high-power objective (4 mm.) is needed occasionally for detailed examination of casts and to distinguish between pus cells and red blood cells. For low-power examination it is not necessary to use a cover slip; it may be desirable to use one when the higher power objective is used. The sub-stage condenser should be lowered and the light cut down by the iris diaphragm until the objects stand out clearly.

III. Recording the Findings.- The frequency of occurrence of the various objects observed should be noted as well as their mere presence. The terminology used may be: occasional, few, many, very many, etc. A uniform technic of examination and of reporting should be followed so that the results of different examinations may be comparable. The same amount of urine should be centrifugalized at the same speed for the same length of time in each case. The supernatant should be poured off to the same degree of completeness, and approximately the same thickness of drop examined.



#### IV. Character of the Urinary Sediments.

There are two classes of sediments: (A) unorganized chemical compounds and (b) organized, formed, morphological entities. The latter are by far the more important as the mere presence of some or a superabundance of others indicates pathology somewhere in the urinary tract provided the specimen has not been contaminated.

A. Unorganized Sediment.- Only rarely has the unorganized sediment clinical significance. Its presence and character depends on metabolic activities of the body which are influenced by innumerable factors, or upon fermentation and decomposition processes occurring in the bladder or in the container after voiding. Unorganized sediments appear as crystals of definite structure that can be readily identified morphologically or as amorphous deposits having a granular, structureless appearance; these latter can be partially identified by solubility or microchemical tests conducted on the slide while under microscopic observation.

In acid urines one may find amorphous, pinkish sediments of urates; brownish, wedge-like, "whetstone", or dumb-bell crystals of uric acid; small dumb-bell or square "envelope" crystals of calcium oxalate; refractile, colorless, six-sided plates of cystine; yellowish, small spheroids of leucine; fine needles of tyrosine; and brownish needles or prisms of hippuric acid.

In neutral urines those already mentioned may occur and in addition alender, pyramidal crystals of neutral calcium phosphate united at their apices to form rosettes.

Alkaline urine may contain white amorphous phosphate deposits; "coffin lid" or feathery crystals of ammonium magnesium phosphate ("triple phosphates"); spheres or dumb-bells or amorphous deposits of calcium carbonate; and dark yellow to brown "cockle burr" crystals of ammonium urate.

#### B. Organized Sediments.

1. Casts.- As the name suggests these are moulded in the tubules of the kidney and are composed of an albuminous material and various types of cells. They vary greatly in size but in almost all instances their sides are parallel and ends rounded or broken off squarely. They may be straight or curved, long or short, but the diameter is usually uniform throughout the length. Casts have been classified according to their microscopical characteristics as hyaline, granular, epithelial, blood, pus, fatty and waxy. The finding of casts in the urine is very important for their presence usually indicates some form of kidney disorder, especially if albumin is also present.

(a) Hyaline casts are found more frequently than any others. They are composed of colorless, homogeneous, slightly refractile material; are usually narrow; cellular elements may be attached to the surface.

(b) Granular casts are usually short and thick. The basic hyaline substance is filled with granules which may be fine or coarse.

(c) Epithelial casts bear on their surface cells from the lining of the kidney tubules. The basic material is hyaline, either granular or non-granular.

(d) Blood casts are made up of red blood cells on a fibrin or hyaline base.

(e) Pus casts are quite rare. They are covered with pus cells on a hyaline base.

(f) Fatty casts may be produced by the deposition of fat globules or fatty acid crystals on a hyaline cast.

(g) Waxy casts are made up of a basic substance similar to that of hyaline casts. As a rule they are longer, larger, and more refractile, with sharper outlines and sometimes of a yellowish color.

2. Cylindroids and Pseudo-casts.- Mucus threads, with or without deposits of unorganized sediment about them may simulate true casts. They are usually rough-edged, larger, tapering or frayed at the ends. Cylindroids are often pale, ribbon-like structures, too long to be casts, with variable widths and small diameters.

3. Leukocytes or Pus Cells.- These are round, mono- or poly-nucleated structures, ordinarily colorless. Adding a drop of dilute acetic acid to the sediment brings out the nucleus. They may be scattered about the field singly or occur in clumps.

4. Erythrocytes or Red Blood Cells.- In fresh urine they appear as biconcave discs becoming compact and crenated in concentrated acid urines, and swollen, colorless, disintegrating faint shadows in dilute alkaline urine. Where less than 12 red blood cells are present per high power field, the benzidine test is likely to be negative.

5. Epithelial cells.- A few squamous cells are usually present. In certain pathological conditions they are greatly increased in number and since the different parts of the urinary tract are lined with different types of epithelium, the number and type of cells present may be of considerable diagnostic value.

6. Spermatozoa are readily identified by the characteristic oval head and long tail.

The illustrations in this section give a general idea of the appearance of the more usually encountered unorganized and organized sediments.



# QUANTITATIVE CHEMICAL EXAMINATION

## I. Albumin.

A. Method adopted by the Committee on Urinary Impairments of the Association of Life Insurance Directors of America.

### 1. Reagents.-

(a) Sulfosalicylic acid. A 3 per cent solution in distilled water.

(b) Permanent Standards.- Dissolve 20 gm. of purest sheet gelatin in 120 to 140 cc. of distilled water at 45 to 55°C. and make up to 200 cc. Add half of the white of an egg and stir it in well. Heat on a water bath for at least thirty minutes after a temperature of 90°C. has been attained. Filter hot through a Whatman No. 4 paper, yielding a perfectly clear, slightly yellow solution. Immediately before use add 0.3 cc. of formalin (40% formaldehyde solution) to each 100 cc. of gelatin solution. Formazin, the material to be suspended in the gelatin is made up as follows: Dissolve 2.5 gm. of urotropin (hexamethylene tetramine) in 25 cc. of distilled water at room temperature. Add this to 25 cc. of 1% hydrazine sulfate solution also at room temperature. Mix, stopper and allow to stand at least fifteen hours. Suspend the white amorphous precipitate uniformly by gently inverting the flask several times. Add 14.5 cc. of the formazin suspension to 100 cc. of the 10 per cent gelatin solution, to which the correct amount of formalin has been added, at 45 to 55°C. and mix thoroughly. This produces a turbidity equivalent to that made by an albumin solution of 0.1 per cent, or 100 mg. in 100 cc., when precipitated by 3 volumes of 3 per cent sulfosalicylic acid. Dilute the stock suspension according to the following table to make the other standards required.

TABLE  
Permanent Albumin Standards

Stock formazin suspension equivalent to 100 mg. al- bumin per 100 cc.	10% clarified gelatin	Value of Standard made.
<u>cc.</u>	<u>cc.</u>	
25.0	26	0.05 per cent or 50 mg.
20.0	30	0.04 per cent or 40 mg.
15.0	35	0.03 per cent or 30 mg.
10.0	40	0.02 per cent or 20 mg.
5.0	45	0.01 per cent or 10 mg.
2.5	55	0.005 per cent or 5 mg.

Pour each standard into a test tube of the same dimensions as those used in making the test with urine. Seal the tubes with waxed stoppers and allow to cool to room temperature. Keep in a well-lighted room. In extremely hot weather, keep in a cool place. If in time they become greenish, exposure to



sunlight will bleach them. There is no appreciable change in turbidimetric value in six to eight months and only a slight change in a year. It is best to replace them after eight months.

2. Procedure.- Pipette 2.5 cc. of urine, cleared by filtration or centrifugalization, into a test tube graduated at 10 cc. and add 3 per cent sulfosalicylic acid to the 10 cc. mark. Invert several times, allow to stand for ten minutes and compare the turbidity with that of the permanent standards. Record the value of the standard most closely matched as the albumin content of the urine.

#### B. Sedimentation Method of Shevsky and Stafford.

1. Reagents.- Tsuchiya's solution is used as the precipitant. Mix 15 gm. of phosphotungstic acid, 50 cc. of concentrated hydrochloric acid and 1000 cc. of 95 per cent ethyl alcohol.

2. Procedure.- The first step is the dilution of the urine. Nephritic urines are usually diluted ten-fold. In urines with very scanty protein content a lesser dilution or none at all will give more exact results. Occasionally a urine is encountered with more than 2.8 per cent of protein which is the maximum that can be determined with a ten-fold dilution. In such a case a fresh sample is diluted twenty-fold and the determination repeated. Of the diluted urine, 4 cc. are measured into a special graduated centrifuge tube (Shevsky - Stafford or McKay tube; A. H. Thomas Co. #3007-A; Eimer and Amend #18368; Fisher Scientific Co. 5-663), the 4 cc. mark on the tube itself serving for the measurement. Tsuchiya's reagent is added to the 6.5 cc. mark. Mix the contents well by inverting the tube several times, allow to stand exactly ten minutes and centrifugalize for exactly ten minutes at 1800 r.p.m. The volume of precipitate is read on the scale, in hundredths of a cc.

3. Calculation.- Grams of protein per liter of urine = cc. of precipitate x 7.2 x dilution, where dilution indicates the number of times the urine was diluted before the sample was measured into the tube.

#### II. Glucose.- Benedict Method.

##### 1. Reagents.

(a) Sodium carbonate, crystals or monohydrated salt..

(b) Pumice or talc.

(c) Benedict's Reagent, Quantitative.

Copper sulfate, U.S.P. crystals ( $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ ) 18.0 gm.

Sodium carbonate, monohydrated. ( $\text{Na}_2 \text{CO}_3 \cdot \text{H}_2\text{O}$ ) 87.0 gm.

(74 gm. of anhydrous  $\text{Na}_2\text{CO}_3$  or 200 gm. of the crystalline salt,  $\text{Na}_2\text{CO}_3 \cdot 10 \text{H}_2\text{O}$ , may be used.)

Sodium or Potassium citrate	200.0 gm.
Potassium thiocyanate	125.0 gm.
Potassium ferrocyanide, 5 per cent solution	5.0 cc.
Distilled water to make	1000.0 cc.

With the aid of heat, dissolve the carbonate, citrate and thiocyanate in enough water to make about 800 cc. of solution. Filter if necessary. Dissolve the copper sulfate separately in about 100 cc. of water, and pour this solution slowly into the first one, with constant stirring. Add the ferrocyanide solution, cool to room temperature and make up to 1000 cc. in a volumetric flask. Of the various salts only the copper sulfate must be weighed with extreme accuracy. Exactly 25 cc. of this reagent are reduced by 50 mg. of glucose.

2. Procedure.- Dilute 10 cc. of clear urine to 100 cc. with water, unless the sugar content is known to be low. Fill a 50 cc. burette with this diluted urine. Measure exactly 25 cc. of the Benedict's reagent into a porcelain evaporating dish, add about 15 gm. of crystalline sodium carbonate (half that amount of the monohydrated salt, or 6 gm. of the anhydrous), and a small amount of pumice or talc. Heat to boiling over a free flame and keep the mixture boiling vigorously during the entire titration. As soon as the carbonate is completely dissolved add the diluted urine from the burette, rapidly at first, until a chalk-white precipitate forms and the blue color begins to fade perceptibly. It is then run in a few drops at a time until the last trace of blue disappears from the solution. Half minute intervals must be allowed to elapse between additions of urine in the final steps of the titration. Water may be added if the mixture becomes too concentrated. The end-point must be determined while the solution is still hot; upon cooling the solution tends to regain a bluish-green tint. With urine, the color at the end-point tends to be a slight yellowish, or yellowish-green due to urinary pigments.

3. Calculation.- When the urine is diluted 1:10, the following formula applies:

$$\frac{0.050}{N} \times 1000 = \text{per cent of glucose in original sample, where } N \text{ is the number of cc. of diluted urine required to reduce 25 cc. of the reagent.}$$
  
In general, 
$$\frac{0.050}{X} \times 100 = \% \text{ glucose, where } X = \text{number of cc. of undiluted urine required for the reduction.}$$

### III. Hydrogen Ion Concentration or pH.

There are numerous methods for determining the hydrogen ion concentration (or pH) of the urine, but the simplest method satisfactory for clinical purposes is that involving the use of nitrazine paper (phenaphthazine).

1. Reagent.- Nitrazine paper, Squibb.

2. Procedure.- With a clean glass rod transfer a drop of urine to the surface of the paper strip and spread evenly by stroking or leave a small drop on the paper. After one minute, compare with the color chart furnished.

The paper may be dipped into the urine three consecutive times and the excess shaken off. Compare after one minute.

3. Results.- The color comparison chart reads from pH 4.5 to 7.5 in 0.5 divisions. It is possible to interpolate between these divisions by estimating the color half way between them. Report the pH as read.





## EXAMINATION OF GASTRIC CONTENTS

The examination of the gastric contents yields information as to the secretory, digestive and motor functions of the stomach and indirectly aids in the diagnosis of conditions that affect the stomach secondarily, such as the demonstration of absence of acidity in pernicious anemia.

The routine examination is concerned chiefly with the secretory response of the stomach to a test meal or to the stimulation supplied by drugs such as histamine. But, in the course of a complete examination, pathological conditions as hemorrhage, new growth or parasites may be disclosed. It is well to keep in mind that the gastric contents are not only supplied by the activity of the stomach, but include a variety of materials swallowed and others that are regurgitated from the small intestines. Pus, blood, bacteria, tissue fragments, etc., from the nose, mouth, pharynx and esophagus find their way into the stomach; similar materials and secretions and excretions of the liver, gall-bladder and duodenum may be regurgitated through the pylorus, and with reversal of peristalsis, the fecal contents of the jejunum and ileum may likewise gain entrance.

The fundus glands of the stomach secrete hydrochloric acid, and the ferments pepsin and rennin which are activated only in the presence of free hydrochloric acid. Lipase is also secreted but it has little activity except on previously emulsified fats such as milk or egg yolk. The pyloric glands contribute an albuminous secretion. The goblet cells, distributed along the entire surface epithelium and along the ducts of the fundus and pyloric glands add mucus.

The hydrochloric acid combines loosely with protein food; this is designated "combined HCl". When all the protein has been converted to acid metaprotein, the additional acid secreted remains uncombined and is designated "free HCl". The pepsin activated by it continues the digestive process. In pathological conditions there may be an absence of hydrochloric acid designated achlorhydria; this is also occasionally seen in normals in the first specimens of the fractional analysis. The absence of "free HCl" does not necessarily mean that no acid is being produced; it may be secreted and neutralized by an excessive regurgitation of the alkaline duodenal juice; this physiological regurgitation is a normal process and may reduce an acidity of 0.4 per cent to as low as 0.15 per cent.

## PHYSICAL EXAMINATION

### I. Macroscopic Examination.

1. Amount.— The capacity of the average adult stomach is 1500 to 1600 cc. The fasting residuum varies between 20 and 100 cc., usually less than 75 cc. with an average of 50 cc. An increase in the residuum may be due to hypersecretion, retention, swallowing of saliva, or duodenal regurgitation. Ordinarily these may be differentiated by noting retained food particles from the previous day's meal in retention cases, or by finding increased amounts of bile and trypsin in regurgitant cases. In true hypersecretion, food



particles, increased amount of bile and duodenal ferments are absent and the juice may show no abnormality except some increased acidity. The amount of juice recovered in one hour after the Ewald type of test meal varies from 50 to 100 cc. Larger amounts, 200 to 300 cc., are found in hypersecretion and hypomotility and excessive amounts, 500 to 600 cc., in dilatation accompanying pyloric stenosis. Amounts less than 20 cc. may be due to incomplete removal, hypermotility or incomplete closure of the pylorus due to chronic inflammation or scarring.

2. Emptying Time.- The normal stomach empties itself completely in several hours at the longest, but the time may vary somewhat with the diet ingested. After the ordinary mixed meal it is emptied in four to seven hours, after the Ewald type in one to three hours. Large quantities of juice obtained after the normal time for emptying indicate a pathological basis.

3. Color.- The gastric juice, as obtained from the fasting stomach, is usually clear, colorless and easily filtered. Over 50 per cent of residuums are faintly bile-tinged, appearing green or yellow due to regurgitation of bile incidental to straining during tube passage.

A bright red color is an indication of fresh bleeding; dark brown or black blood is encountered in older bleeding, the blood having been acted on by the hydrochloric acid, producing a "coffee-ground" appearance. Blood in small amounts is especially characteristic of carcinoma; in larger amounts, of esophageal varices or ulcers and in massive amounts, up to 1000 to 2000 cc., of ruptured varices or aneurysm or sudden ulceration into a large gastric vessel. The presence of blood, if gross, is easily detected, although tomatoes, grape skins, etc., may give confusing reddish or dark flecking. Occasionally it will be necessary to differentiate gastric from pulmonary hemorrhage; in the former the vomitus is usually acid, the color is dark red or brown, and clots are present; in the latter the expectoration is usually alkaline, brighter in color, frothy and usually mixed with mucus. In all cases it is wise to confirm the gross impression by performing a chemical test for blood.

4. Layering.- The residuum from a fasting stomach normally shows no sediment. After the Ewald test diet the one-hour specimen forms two layers - the lower consisting of food residue, the upper of clear or slightly cloudy grayish-white or faintly yellow fluid. Ordinarily the proportion of solid to fluid is 1 to 1 or 1 to 2; larger proportions of solids suggest delayed emptying or hyposecretion; larger proportions of fluid indicate hypersecretion. The condition of the food particles gives a rough indication of the digestive efficiency. Normally the breadstuffs forming the bulk of the Ewald type meal are acted on by the ptyalin of the saliva and appear in the lower layer as a pureed mass; in hyperacidity this enzyme is quickly destroyed so that the breadstuff fragments appear incompletely digested. In cases of pyloric stenosis three layers may be noted - at the bottom fine, starch-like material, midway a thick layer of cloudy fluid and on top a foamy layer indicating gaseous fermentation.

5. Odor.- The residuum of the normal fasting stomach is practically odorless. The normal one-hour contents after the Ewald test meal yield a sweet



odor of moist bread. Sour odors, due to acetic acid formation in the course of carbohydrate fermentation, are noted in the contents after longer retention. Rancid odors of butyric acid accompanying lactic acid formation, and protein decomposition occur in retention usually with decreased or absent hydrochloric acid. Lactic acid itself is odorless. Putrid odors may accompany carcinomatous ulceration of the stomach due to the discharge into the stomach of the contents of ruptured abscesses of the surrounding tissues. A fecal odor accompanies the discharge of the contents of the small bowel into the stomach during the reversal of peristalsis accompanying intestinal obstruction.

### TEST MEALS

I. Retention or Motor Meal.- It is the purpose of such meals to secure information regarding gastric motility or retention. The meal contains 4 ounces of boiled string-beans and 4 ounces of rice and is substituted for the regular evening meal on the day preceding that on which the gastric test is to be made. Instead of employing a special meal, the regular supper can be supplemented by adding 4 stewed prunes or 30 raisins, or raspberry preserve. The idea is to include articles of diet leaving residues that can be readily recognized in the gastric residuum of the fasting stomach the following morning.

II. Functional Meals.-1. Ewald Type.- The purpose of any functional test meal is to stimulate gastric secretion, and to estimate the functional response of the gastric mucosa by the determination in samples of the gastric contents of the production of acid and gastric ferments. A variety of test meals have been prepared. The Ewald meal or some modification is the common test meal and is used in both the older Topfer single sample method and the newer Rehfuess fractional method of gastric analysis. It consists of 2 slices of toasted bread (35 gm.) and 8 ounces (250 cc.) of water or tea without milk or sugar. Water is to be preferred if tests for the detection of occult blood are to be used, as the tannic and gallic acid of tea interfere with blood tests. The use of toast introduces some lactic acid into the stomach, so that if a test for lactic acid in the contents is particularly desired, a modified Ewald meal or a Boas meal, consisting of oatmeal gruel containing no lactic acid, is used. The gruel is prepared by boiling down to 500 cc. 1 liter of water to which 2 tablespoonfuls of oatmeal are added and straining this concentrate through coarse muslin. The Mayo Clinic modification of the Ewald meal consists of 8 arrowroot cookies and 400 cc. of water; it contains no lactic acid.

2. Clear Fluid Type.- This type of test meal, or rather test drink, is employed chiefly in Europe. Bergheim, in this country, showed that ordinary tap water produced as much stimulation as the Ewald type meal. The Bergheim water meal consists of 400 cc. of ordinary tap water at room temperature. The alcohol test meal consists of 300 cc. of 5 per cent pure ethyl alcohol solution (15 cc. of absolute alcohol brought up to 300 cc. by adding water). The caffeine test meal consists of 400 cc. of water containing 0.2 gm. of pure caffeine. Usually these clear fluid meals are colored by adding 3 drops of 2 per cent solution of methylene blue (methylthionine chloride, U.S.P.), which aids in following the propulsion of the meal out of the stomach. As the stomach empties, the blue color diminishes with each subsequent specimen examined in the fractional method, until colorless specimens are obtained,

indicating complete emptying of the stomach. It also helps in recognizing the regurgitation of alkaline bile or colorless duodenal contents by the change of the blue to a characteristic greenish hue. The clear fluid meals permit more accurate determination of food residue, blood, lactic acid and bile.

The Ewald type is the most satisfactory for routine examination by the Töpfer method. The clear fluid meals, when used in single extraction methods, leave the stomach too quickly, the stomach being empty after forty-five minutes. In the fractional method of gastric analysis the clear fluid meals are advantageous in that they can be readily extracted through the small tubes employed; the gastric contents are clear and titrations can be more easily read and no preliminary filtration of the contents is necessary.

### ROUTINE CHEMICAL ANALYSIS

Two main methods are employed: (1) The method of Töpfer in which a test meal of the Ewald type is given as breakfast, and a single withdrawal of all the contents is made one hour after the ingestion of the test meal. These contents are then examined chemically by Töpfer's method. (2) The fractional method of Rehfuess, in which a retention meal is given for supper the previous evening, and, after a twelve-hour fast, the fasting residuum is aspirated, the tube left in place, a test meal of Ewald or clear fluid type given, and sample specimens withdrawn every fifteen minutes for one or more hours until the stomach is empty. Each fraction is examined for total and free acidity; other tests may be made when indicated. The fractional method follows the cycle of gastric digestion and secretion, allowing the plotting of curves which permit better interpretation, especially of acid secretion.

I. Method of Töpfer.- Measure and record the volume of the sample. Strain through cheesecloth and place 10 cc. of the coarsely filtered fluid in each of three beakers or porcelain dishes labeled I, II and III. If sufficient contents are not obtained, use 5 cc. and calculate accordingly.

Normally 50 to 100 cc. are recovered. In hypersecretion or defective motility, 200 to 300 cc. may be recovered. Excessive volumes of 500 to 600 cc. indicate gastric dilatation due to pyloric stenosis, benign or malignant.

#### 1. Reagents.

(a) Phenolphthalein Indicator.- Dissolve 0.5 gm. of phenolphthalein in 100 cc. of 50 per cent ethyl alcohol.

(b) Töpfer's Reagent.- Dissolve 0.5 gm. of p-dimethylaminoazobenzene in 100 cc. of 95 per cent ethyl alcohol.

(c) Alizarin Red Indicator.- Dissolve 1 gm. of sodium alizarin monosulfonate in 100 cc. of water.

(d) Sodium Hydroxide 0.1 N solution.



## 2. Procedure.

(a) Total Acidity.— This includes free hydrochloric, combined hydrochloric, organic acids and acid salts.

To the 10 cc. sample of gastric contents in beaker I, add 1 drop of phenolphthalein indicator (which is colorless in the presence of acid). Add 0.1 N sodium hydroxide solution from a burette until a faint pink is produced that persists for two minutes. The number of cubic centimeters used is multiplied by 10 to obtain the number of cubic centimeters of 0.1 N hydroxide necessary to neutralize 100 cc. of gastric fluid. The value obtained expresses the total acidity. This can be converted into terms of hydrochloric acid by multiplying by 0.00365, which is the equivalent value of 1 cc. of 0.1 N sodium hydroxide in grams of hydrochloric acid.

(b) Free Hydrochloric Acid.— This is hydrochloric acid not combined with protein material.

To sample II add 2 to 4 drops of Töpfer's reagent and titrate with 0.1 N sodium hydroxide until the initial red color becomes salmon-pink. If there is an initial yellow color on adding the indicator no free acid is present. The number of cc. of sodium hydroxide solution used, multiplied by 10, gives the value for 100 cc. of the gastric juice. Töpfer's reagent occasionally gives a red color in the absence of HCl due to large increase in the organic acids, especially when lactic acid is over 1 per cent and albumoses are present. In case the amount of gastric juice is small, this same specimen may be used to determine the total acidity. After the end point is reached for free HCl add 2 drops of phenolphthalein indicator and continue the titration with 0.1 N sodium hydroxide until the persistent pink end point of total acidity is reached. The number of cc. of hydroxide used in the determination of the free HCl, plus the additional cc. necessary to complete the titration with phenolphthalein, is multiplied by 10, giving the value of the total acidity.

(c) Free Acidity.— This includes hydrochloric acid in the free state, organic acids and acid salts, but does not include the combined hydrochloric acid.

To sample III add 1 to 3 drops of sodium alizarin sulfonate solution. Titrate with 0.1 N sodium hydroxide solution. As the hydroxide is added the initial tinge of yellow changes to red. The end point is indicated by a distinct violet color. The number of cubic centimeters of hydroxide used, multiplied by 10 gives the free acidity value. Töpfer states that alizarin is sensitive to all acidity except combined HCl.

(d) Combined Hydrochloric Acid.— This value is obtained by subtracting the value obtained for free acidity from that of the total acidity. Cases are seen where there is no free hydrochloric, but much combined acid, indicating that acid has been secreted but has combined with the food protein.

(e) Organic Acids and Acid Salts.— This value is computed by subtracting the value of free HCl from that of the free acidity, the remainder expressing this value.



3. Results.- Total acidity has a wide range normally, being equivalent to 75 - 100 cc. of 0.1 N sodium hydroxide per 100 cc. of gastric contents. Of this approximately 50 per cent is due to free hydrochloric acid, 25 per cent to combined hydrochloric, and 25 per cent to organic acids and acid salts. In pathological conditions wide variations may occur from low or absent total acidity to very high acidity with correspondingly high free hydrochloric acid.

II. Fractional Method of Rehfuess.- The patient is instructed to eat, with the evening meal on the day preceding the test, some food article which will leave an easily identifiable residue if there is gastric retention. Such articles as several prunes, 2 dozen raisins in rice pudding, or raspberry preserve, answer this purpose. Nothing is to be taken by mouth after 9 P.M. The following morning with the patient in the basal state the test is begun.

A narrow, flexible gastroduodenal tube of the Rehfuess type is swallowed for 20 to 22 inches from the lip margin; check its position by aspirating with a 50 cc. Luer syringe until free withdrawal of contents is obtained. When the tube is in the stomach, withdraw by aspiration all the contents, measure the volume, and save the specimen for examination. Maintain the position of the tube by strapping it to the angle of the mouth with adhesive; between withdrawal of the fractions of gastric juice, keep its free end clamped.

An Ewald type meal is given, or a simple water meal, which is easier to swallow past the tube; the tube remains in situ until the test is completed. Record the time at which the last of the meal is swallowed. Any saliva that forms is to be expectorated to avoid its diluting effect on the gastric contents.

Samples of about 5 to 10 cc. are withdrawn at exactly fifteen-minute intervals, terminating each withdrawal by forcing a few cc. of air down the tube to free it for the next withdrawal. To insure a mixed sample of juice the contents may be aspirated and forced back several times before a sample is taken. Each sample is kept separate and marked with the time taken or the number of the specimen.

In the definite period test the fractions are withdrawn every fifteen minutes for two hours. The last specimen should represent all the gastric juice remaining in the stomach at two hours. To insure complete emptying, the patient turns from side to side and from back to stomach; aspiration being made with the patient in these various positions. By forcing a few cc. of air down the tube, gurgling sounds will be heard with a stethoscope if there is some juice remaining. Another method is to withdraw all the juice possible and then irrigate the stomach through the tube with 200 cc. of water, observing the presence or absence of food particles in the washings.

In the total secretion test, the fifteen-minute fractions are continued until the stomach is completely empty in order to judge the motility of the stomach. In hypomotility the test is thus prolonged for three hours or more.

The usual physical examinations are made of each fractional sample. Chemical examination is confined to total acidity and free hydrochloric acid as a

rule because the individual samples are limited in amount. Strain the fasting sample and each fraction separately through gauze or cheesecloth.

1. Reagents.- The same as in Töpfer's Method except that 0.01 N sodium hydroxide is used instead of 0.1 N.

## 2. Procedure.

(a) Total Acidity.- One cubic centimeter of the filtrate and 15 cc. of water are placed in a porcelain evaporating dish. One drop of phenolphthalein solution is added, and a titration is made, using 0.01 N sodium hydroxide until a faint pink lasting for two minutes indicates the end point. Calculation: The number of cc. of 0.01 N sodium hydroxide required to neutralize 1 cc. of the sample, multiplied by 10 gives the number of cc. of 0.1 N hydroxide needed to neutralize 100 cc. of the gastric contents.

(b) Free Hydrochloric Acid.- (1) Töpfer Method.- Place 1 cc. of the strained specimen and 15 cc. of water in a porcelain evaporating dish. Add 1 to 2 drops of Töpfer's reagent; if free HCl is present a red or orange color develops. Titrate with 0.01 N Sodium hydroxide solution until an orange-yellow color appears (end color is more definitely yellow than orange). The calculation is the same as in total acidity.

(2) Sahli Method.- This requires more time but gives a sharper end point. It is based on the liberation of iodine from the reagent employed, in the presence of free HCl. The iodine is titrated with sodium thiosulfate, using a starch indicator.

Place 1 cc. of the strained sample and 10 cc. of water in a porcelain evaporating dish. One cubic centimeter of Sahli's reagent (a mixture of equal parts of a 48 per cent solution of potassium iodide and an 8 per cent solution of potassium iodate) is added; allow the mixture to stand five minutes. Titrate with 0.01 N sodium thiosulfate until only a faint yellow of the liberated iodine remains. Add 0.5 cc. of 1 per cent soluble starch solution; the mixture turns blue; continue titration until the blue disappears.

The total number of cc. of 0.01 N sodium thiosulfate used in the titration of 1 cc. of gastric specimen is equivalent to the number of cc. of 0.01 N sodium hydroxide necessary to neutralize the free HCl in 1 cc. of gastric contents. This value multiplied by 10 represents the number of cc. of 0.1 N sodium hydroxide necessary to neutralize 100 cc. of stomach contents.

3. Results.- It is customary to plot the results of the acidity determinations as a curve on special forms provided for the purpose or on the reverse of the regular form. The graph is made by plotting the cc. of 0.1 N sodium hydroxide required to neutralize 100 cc. of gastric contents against the time in minutes or hours. For examples of normal and abnormal graphs, see the reference books listed at the end of this chapter.



III. Lactic Acid.- Lactic acid is a product of carbohydrate fermentation by bacteria. In health it is not present at the height of digestion. Small amounts may be introduced with the food and may be found in the first fractional specimens. It is most often present with stagnation of the gastric contents associated with deficient HCl. The combination of lactic acid, hypochlorhydria and decreased motility occurs most often in gastric carcinoma.

#### A. Phenol-Ferric Chloride Test (Uffelmann).

1. Reagent.- Add 10 per cent ferric chloride solution to a 1 per cent aqueous phenol solution until an amethyst color develops.

2. Procedure.- To 5 cc. of reagent add 5 cc. of strained gastric juice. To another 5 cc. portion add a few drops of dilute hydrochloric acid as a control.

3. Result.- Lactic acid produces a canary-yellow color. The reagent will detect 0.01 per cent of lactic acid. Hydrochloric acid discharges the amethyst color, leaving the solution colorless. If the gastric juice contains much free HCl the value of the test is decreased. Other organic acids give results similar to lactic.

#### B. Mercuric Chloride-Ferric Chloride Test (MacLean).

1. Reagent.- Dissolve 5 gm. of ferric chloride in a mixture of 100 cc. of saturated aqueous solution of mercuric chloride and 1.5 cc. of concentrated hydrochloric acid.

2. Procedure.- Place 5 cc. of water in a test tube as a control. In another tube place 5 cc. of gastric contents. To each add 5 drops of reagent.

3. Result.- A reddish color indicates the presence of lactic acid.

#### C. Ether-Ferric Chloride Test (Strauss).

In this test the lactic acid is extracted from the gastric contents with ether, making a more satisfactory test since hydrochloric acid, protein digestion products and other disturbing factors are eliminated.

1. Reagents.- (a) Ether. (b) Ferric Chloride, 10 per cent aqueous solution.

2. Procedure.- Place 5 cc. of strained gastric contents in a small separatory funnel. Add 20 cc. of ether and shake thoroughly. Let stand until the ether layer has separated, then run out the layer of gastric juice and all but the final 5 cc. of ether. To this add 20 cc. of distilled water and 2 drops of the ferric chloride solution. Shake the mixture gently.

3. Result.- When lactic acid is present in a concentration of 0.05 per cent a slight greenish color develops. If the concentration is 0.1 per cent, the color is an intense yellow. The color is due to ferric lactate.



#### IV. Occult Blood - Benzidine Test.

This test is a very sensitive one provided the reagents are satisfactory. Different lots of benzidine vary greatly in sensitivity and hydrogen peroxide solution rapidly loses its strength. For this reason it is always advisable to set up a positive control using water with an extremely minute amount of blood added, such as would adhere to the tip of an applicator.

1. Reagents.- (a) Benzidine crystals.- Prepare a saturated solution in glacial acetic acid. If kept in a brown bottle in a dark place this solution will keep fairly well. Many prefer to make the solution just before use by adding the amount of crystals picked up on the point of a knife blade to 5 cc. of glacial acetic acid and warming gently to effect solution.

(b) Acetic Acid, glacial.

(c) Hydrogen Peroxide (The usual solution containing 3 per cent  $H_2O_2$ ). Test the peroxide before use by adding a few drops of potassium dichromate solution and a few drops of concentrated sulfuric acid. If the peroxide is still active, a blue color will develop.

#### 2. Procedure for Gastric Contents.

(a) Direct Test.- To 3 cc. of the saturated solution of benzidine in glacial acetic acid add 2 cc. of the gastric contents and mix thoroughly. Add 1 cc. of hydrogen peroxide solution.

(b) Confirmatory Test.- If fat is present, make the gastric contents slightly alkaline with sodium carbonate or sodium hydroxide solution. Extract in a separatory funnel with an equal amount of ether. Discard the ether extract. Make the residue acid with acetic acid and extract with ether. Evaporate the ether extract to dryness, using a water bath which has been heated to boiling and the flame then turned off. Add 1 cc. of water, stir to dissolve the residue, then add a few drops of benzidine solution and a drop or two of hydrogen peroxide.

(c) Results.- If blood is present a green to deep blue color, depending on the amount of blood, will form on adding the peroxide, in either test. Too much benzidine solution or too much peroxide interfere with the delicacy and accuracy of the test.

#### 3. Procedure for Feces.

(a) Regular Test.- Make a thin fecal suspension in about 5 cc. of water. Shake with 5 cc. of ether to remove fat. Discard the ether extract. Acidify the residue with acetic acid and again extract with 5 cc. of ether. Evaporate the ether extract and test as above.

(b) Slide Test.- Smear a little of the feces on a microscopic slide. Pour over it the reagent made by dissolving a knife-tip of benzidine in 2 cc. of glacial acetic acid, to which is then added 1 to 1.5 cc. of peroxide solution.

(c) Results.- A green to deep blue color shows the presence of blood. With the slide test, the smear turns blue without any misleading green tints from the fluid.

#### 4. Procedure for Urine.

(a) Direct Test.- To 2 cc. of urine, or to the centrifugalized urinary sediment mixed with 2 cc. of water, add 3 cc. of benzidine solution and 1 cc. of peroxide solution.

(b) Confirmatory Test.- Add a drop or two of glacial acetic acid to 10 cc. of urine. Extract with 5 cc. of ether. Evaporate the ether extract and test as shown under the confirmatory test for gastric contents.

(c) Results.- A green to deep blue color indicates the presence of blood.

Note: The benzidine test is sensitive to 1 part of blood in 3,000,000.

A modification of the tests above consists of wetting a filter paper, suspended by a clip, with the gastric contents, fecal suspension, or urine sediment. When partly dry, allow a few drops of the reagent prepared as for the slide test with feces, to flow across the material on the filter paper. The blue color of a positive test shows up clearly and promptly.



## CHEMICAL EXAMINATION OF THE BLOOD

## I. Preparation of Protein-free Blood Filtrate (Folin and Wu).

## 1. Reagents.

- (a) Sodium Tungstate: 10 per cent aqueous solution.
- (b) Sulfuric Acid: 0.66 N. To 66 cc. of 1 N sulfuric acid add 33 cc. of distilled water.
- (c) Sulfuric Acid: 10 per cent.
- (d) Benzoic Acid: 0.25 per cent aqueous solution.

The protein precipitation described below need not be made in a volumetric vessel; the procedure is itself volumetric, and is, therefore, applicable to any measured quantity of blood. Ten cc. of blood gives ample filtrate for a complete analysis, though more may be used if desired; if no determinations must be repeated, or if all the constituents are not to be determined, less blood is sufficient. The fact that the protein precipitation is done volumetrically makes for several advantages; it not only allows use of all of a small sample of blood, but it gives a filtrate, which regardless of the initial quantity of blood used, is itself 10 per cent blood. Thus, no matter what amount of blood be taken at first, 10 cc. of the filtrate corresponds to 1 cc. of blood, 5 cc. of filtrate to 0.5 cc. of blood, and so on. This latter fact considerably simplifies the calculations.

Where it is desired to send a specimen to a distant laboratory for analysis, whole blood cannot be shipped as it would have decomposed before arriving at its destination. Instead, the blood is drawn in the usual manner, and a protein-free blood filtrate prepared as described below, except that 7 volumes of 0.25 per cent benzoic acid are substituted for the 7 volumes of distilled water customarily used. This filtrate will keep for a considerable period and may be used for the following determinations: Sugar, non-protein nitrogen, urea nitrogen, creatinine, uric acid and chlorides.

2. Procedure.— Transfer a measured amount, which is "1 volume", of oxalated blood to a flask having a capacity of 15 or 20 times that of the volume taken. Dilute the blood with 7 times its volume, i. e., with 7 volumes of distilled water and mix. If the blood is to be shipped to a distant laboratory for analysis the blood is diluted with 7 volumes of 0.25 per cent benzoic acid instead of the 7 volumes of distilled water. Allow to stand until laking is complete. With a pipette add 1 volume of a 10 per cent solution of sodium tungstate and mix. With another pipette add, with shaking, 1 volume of 0.66 N sulfuric acid. Close the flask with a rubber stopper and give a few vigorous shakes. If the conditions are right, hardly a single air bubble will form as a result of the shaking. (If benzoic acid is used, considerable frothing occurs.)

When blood is properly coagulated, the color of the coagulum gradually changes from pink to dark brown; if this change does not occur, even after standing fifteen or twenty minutes, the coagulation is incomplete, due usually to too much oxalate or citrate.



In such an emergency, the sample may sometimes be saved by the cautious addition of 10 per cent sulfuric acid; add the acid drop by drop, shaking vigorously after each addition, and allowing the mixture to stand for a few minutes before adding more, until coagulation is complete.

Pour the mixture on a filter large enough to hold the entire contents of the flask, and cover with a watch glass. If the filtration is begun by pouring the first few cc. of the mixture down the double portion of the filter paper, the filtrate is almost invariably as clear as water from the first drop; if the first portion is not clear, it may have to be returned to the filter.

The only probable sources of error in the above procedure lie in the improper tungstate or sulfuric acid solutions. The sodium tungstate may contain chlorides or too much carbonate. While the sodium tungstate may be purified by recrystallization from alcohol it is preferable and more economical to purchase a tungstate of proven purity. Most of the larger manufacturers of chemicals now prepare a special sodium tungstate for use in the preparation of blood filtrate, which will yield the desired result.

The amount of sulfuric acid used in the precipitation is intended to set free the whole of the tungstic acid and with about 10 per cent excess and to neutralize the carbonate usually present in commercial tungstates. A greater excess of acid must not be used, as a large part of the uric acid will be lost by such procedure; the volumetric sulfuric acid solutions must, therefore, be accurately made and used.

## II. Determination of Non-protein Nitrogen (Folin and Wu).-

1. Reagents.-(a) Sulfuric-phosphoric Acid Digestion Mixture.- Mix 300 cc. of phosphoric acid, syrupy (about 85 per cent  $H_3PO_4$ ) with 100 cc. of concentrated sulfuric acid. Transfer to a tall cylinder, cover well to exclude ammonia, and set aside for sedimentation of calcium sulfate. This sedimentation is very slow, but in the course of a week or so the top part is clear, and 50 to 100 cc. can be removed by means of a pipette. If this cannot be done, rapid centrifugalization will yield a perfectly clear solution. To 100 cc. of the clear acid mixture add 10 cc. of a 6 per cent copper sulfate solution, and 100 cc. of distilled water.

(b) Standard Nitrogen Solution.- A concentrated stock solution is prepared by dissolving 4.716 gm. of pure, dry ammonium sulfate in 1 liter of 0.2 N sulfuric acid. This solution contains 1 mg. of nitrogen per cc. and the solution for use in the color comparison is made by diluting 10 cc. of this solution up to 100 cc. with 0.2 N sulfuric acid. In this last dilution 1 cc. contains 0.1 mg. nitrogen.

(c) Nessler's Solution.- Dissolve 22.5 gm. iodine in 20 cc. of water containing 30 gm. of potassium iodide. After solution is complete, add 30 gm. of metallic mercury and shake vigorously so as to break up the mercury into globules, cooling the mixture from time to time by immersing the flask in cold water. Continue until the supernatant has lost all color due to iodine. Decant the supernatant from the excess mercury and test for free iodine by adding a drop or two of the solution to 1 cc. of a 1 per cent starch solution.



If the starch test for iodine is negative, add a few drops of an iodine solution of the same concentration as that above, until a faint excess of iodine can be detected by the starch test. Dilute the double iodide solution to 200 cc. with distilled water and mix well. Prepare accurately a 10 per cent sodium hydroxide solution from a saturated solution of sodium hydroxide which has been allowed to stand until all carbonates have settled out. To 975 cc. of this 10 per cent hydroxide solution, add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to settle.

2. Procedure.- Introduce 5 cc. of the protein-free blood filtrate corresponding to 0.5 cc. of blood, into a dry, 200 x 25 mm. test tube graduated at 35 cc. These tubes must be made of "Pyrex" glass. Add 1 cc. of the sulfuric-phosphoric acid digestion mixture, and boil vigorously over a micro-burner until the characteristic, dense, acid fumes begin to fill the test tube, which is usually in from three to seven minutes. If the test tube is held in a slightly inclined position, and the heating begun by applying the flame of the micro-burner at the side of the tube and just below the top of the contained mixture, no bumping will occur; as the mixture begins to boil, the flame can be applied lower down, and finally, under the bottom of the tube. Unless, this method of heating is followed, bumping is likely to be troublesome, and may even result in the loss of a part or all of the preparation.

When the sulfuric acid fumes are unmistakable, cut down the flame so that the contents of the tube are just visibly boiling, and close the mouth of the test tube with a small watch glass or funnel.

Continue the heating very gently for two minutes from the time the fumes begin to be unmistakable, even if the solution has become clear and colorless at the end of twenty to forty seconds. If the oxidation is not visibly finished at the end of two minutes, the heating must be continued until the solution is nearly colorless.

Allow the contents to cool for seventy to ninety seconds, and then add 15 to 25 cc. of distilled water; cool further, approximately to room temperature, and add distilled water to the 35 cc. mark. Occasionally there is formed a heavy white precipitate, probably silicates; this may settle out, or can be readily centrifugalized or filtered out, after Nesslerization, and just before reading against the standard.

When the unknown has been prepared, a standard for comparison is made as follows: Place 3 cc. of the standard nitrogen solution, containing 0.3 mg. of nitrogen, in a 100 cc. volumetric flask; add 2 cc. of the sulfuric-phosphoric acid digestion mixture referred to above, and then about 50 cc. of distilled water.

Then add, to the unknown 15 cc., and to the standard 30 cc. respectively, of the Nessler's solution; fill the standard to the mark with distilled water; mix each thoroughly by inverting several times and compare in the colorimeter. It is essential that the unknown and the standard should be Nesslerized at approximately the same time.

3. Calculation.- The reading of the standard, usually 20 mm., multiplied



by 30, and divided by the reading of the unknown, gives the non-protein nitrogen in mg. per 100 cc. of blood.

### III. Determination of Urea Nitrogen (Urea)

1. Reagents.- (a) Urease Paper.- Transfer 30 gm. of jackbean meal to a 200 cc. flask, add 100 cc. of dilute ethyl alcohol (30 cc. of 95 per cent ethyl alcohol diluted to 100 cc.) and 1 cc. of the buffer described in (b) below. Stopper and shake vigorously for fifteen minutes. Transfer to centrifuge tubes, close the mouths of the tubes with tinfoil and centrifugalize for thirty minutes. Transfer the supernatant to a flat-bottomed dish and take up at once on strips of filter paper such as is used in the preparation of amboceptor paper (Schleicher and Schull's No. 597). Suspend the papers from a wire or rack by means of paper clips and allow to dry overnight in an incubator at 37.5°C. As soon as dry cut into pieces about 1 x 2.5 cm. and preserve in wide-mouthed dark glass bottles. Urease so prepared will retain its activity for at least six months.

(b) Acetate Buffer.- Dissolve 15 gm. of crystallized sodium acetate in a 100 cc. volumetric flask with 50 to 75 cc. of water. Add 1 cc. of glacial acetic acid, dilute to volume, and mix.

(c) Standard Nitrogen Solution.- Prepare a stock solution by dissolving 4.716 gm. of pure, dry ammonium sulfate (Item 10680 Ammonium Sulfate ACS, for nitrogen standard - Medical Department Supply Catalogue) in 0.2 N sulfuric acid and make up the volume to 1000 cc. This solution contains 1 mg. nitrogen per cc. For use, dilute 10 cc. of this stock solution to 100 cc. with 0.2 N sulfuric acid. One cc. of this dilution contains 0.1 mg. of nitrogen.

(d) Nessler's Solution.- Dissolve 22.5 gm. iodine in 20 cc. of water containing 30 gm. of potassium iodide. After solution is complete, add 30 gm. of metallic mercury and shake vigorously so as to break up the mercury into globules, cooling the mixture from time to time by immersing the flask in cold water. Continue until the supernatant has lost all color due to iodine. Decant the supernatant from the excess mercury and test for free iodine by adding a drop or two of the solution to 1 cc. of a 1 per cent starch solution. If the starch test for iodine is negative, add a few drops of an iodine solution of the same concentration as that above, until a faint excess of iodine can be detected by the starch test. Dilute the double iodide solution to 200 cc. with distilled water and mix well. Prepare accurately a 10 per cent sodium hydroxide solution from a saturated solution of sodium hydroxide which has been allowed to stand until all carbonates have settled out. To 975 cc. of this 10 per cent hydroxide solution, add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to settle.

(e) Gum acacia, -5 per cent solution. This solution may be preserved by adding 3 cc. of the potassium mercuric iodide solution made for Nessler's solution to each 100 cc. of gum solution. Keep the gum solution in a tall cylinder where the precipitate which forms can settle out.

2. Procedure.- Place 5 cc. of the blood filtrate in a test tube graduated at 25 cc.; to a similar tube add 1 cc. of the standard nitrogen solution containing 0.1 mg. nitrogen and dilute to 5 cc. with distilled water. To both

standard and unknown add 2 drops of the acetate buffer mixture and a piece of urease paper. Stopper and set aside for thirty minutes at room temperature, during which time the tubes are frequently shaken to set free the urease from the paper. At the end of this period add 1 cc. of gum acacia solution to each tube and dilute to about 20 cc. with distilled water. Then to each tube add 2 cc. of Nessler's solution, dilute to the 25 cc. mark and compare in the colorimeter.

The protection against precipitation afforded by the gum acacia is of limited duration and therefore the reading in the colorimeter must be made at once upon the addition of the Nessler's solution to the unknown. No precipitation or change will occur in the standard, consequently a series of unknowns may be read against the same standard provided that each unknown is Nesslerized and diluted to the 25 cc. mark individually just before matching.

3. Calculation.— The reading of the standard, usually 10 mm., multiplied by 20 and divided by the reading of the unknown, gives the urea nitrogen in mg. per 100 cc. of blood. If it is desired to convert this figure to that for urea, multiply by 2.143.

IV. Note.— Blood Urea Clearance.— See Laboratory Methods, U. S. Army, 4th Edition, 1935.

V. Determination of Creatinine (Folin and Wu).

#### 1. Reagents.—

(a) Picric Acid, Saturated Solution.— Most of the U.S.P. picric acid furnished produces considerable color with alkali, which is very undesirable. Certain chemically pure supplies of picric acid may be used without purifying. The grade usually supplied may be purified by various methods, of which the simplest is by recrystallization from acetic acid.

Dissolve 100 gm. of picric acid, previously dried at 80-90°C., in 150 cc. of glacial acetic acid in an Erlenmeyer flask with the aid of heat. Continue the heating on an electric hot plate until the solution boils. Filter hot through a fluted filter paper in a dry funnel which has been heated previously. Collect the filtrate in a dry beaker and cover with a watch glass. Let stand overnight at room temperature. If crystallization does not occur, seed with a small crystal of pure picric acid. After crystallization is complete, filter through a Büchner funnel, by suction, using a hardened filter paper. Wash in the funnel with about 35 cc. of cold glacial acetic acid. Suck as free from acid as possible and dry at 80-90°C., with occasional stirring, until free from the odor of acetic acid. Conduct all operations in a good current of air. Weigh the purified, dried picric acid and add 10 per cent by weight of distilled water. Picric acid containing this amount of water is perfectly safe, while dry picric acid is very explosive. Since a saturated solution in water is used, the added water makes no difference.

(b) Sodium Hydroxide, 10 Per Cent Solution

(c) Standard Creatinine Solution.— A stock solution is first prepared



by dissolving 1.6106 gm. of creatinine zinc chloride in 1 liter of 0.1 N hydrochloric acid. The working standard is prepared by diluting 3 cc. of the stock standard to 500 cc. with 0.01 N hydrochloric acid. Transfer to a bottle and add 4 or 5 drops of toluene or xylene. Five cc. of this solution contains 0.03 mg. of creatinine.

2. Procedure.— Place 10 cc. of the protein-free blood filtrate, corresponding to 1 cc. of blood, in a small flask, or in a test tube. Place 5 cc. of the standard creatinine solution, containing 0.03 mg. of creatinine, in another small flask, and dilute to 20 cc. with distilled water. Place 25 cc. of a saturated solution of picric acid in another small flask and add 5 cc. of a 10 per cent solution of sodium hydroxide, mixing thoroughly. Then add 5 cc. of the alkaline picrate solution, freshly prepared as above, to the blood filtrate, and 10 cc. to the diluted creatinine solution.

Let stand eight to ten minutes and compare in the colorimeter. The readings should be completed within fifteen minutes from the time the alkaline picrate solution was added to the filtrate and standard.

The creatinine standard solution is so made that 5 cc. contain 0.03 mg. of creatinine, and this amount plus 15 cc. of water, represents the standard needed for the vast majority of human bloods, for it covers the range from 1 to 2 mg. per 100 cc. of blood. In the case of bloods representing retention of creatinine take 10 cc. of the standard plus 10 cc. of water, which covers the range from 2 to 4 mg. of creatinine per 100 cc. of blood; or 15 cc. of the standard plus 5 cc. of water, by which 4 to 6 mg. per 100 cc. of blood can be estimated. By taking the full 20 cc. volume from the standard solution, at least 8 mg. can be estimated; but when working with such bloods, it is better to substitute 5 cc. of the blood filtrate plus 5 cc. of water for the usual 10 cc. of filtrate.

3. Calculation.— The reading of the standard, usually 20 mm., multiplied by 1.5, 3, 4.5, or 6 depending upon whether 5, 10, 15 or 20 cc., respectively, of the standard solution were used, and divided by the reading of the unknown, gives the creatinine in mg. per 100 cc. of blood.

When the amount of the blood filtrate available for the creatinine determination is too small to permit a repetition, it is advantageous to start with more than one standard. If, however, a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution, using for such dilution a picrate solution first diluted with 2 volumes of water; so as to preserve equality between the standard and the unknown in regard to the concentration of picric acid and sodium hydroxide.

## VI. Determination of Uric Acid (Benedict).—

1. Reagents.—(a) Standard Solution of Uric Acid.— A stock is first prepared. Transfer exactly 1 gm. of uric acid to a small funnel on a liter volumetric flask. Transfer from 0.45 to 0.5 gm. of lithium carbonate to a 300-cc. beaker, add 150 cc. of distilled water and heat to 60°C., shaking or

stirring until all the carbonate has dissolved. With the hot carbonate solution rinse the uric acid into its flask and shake. The uric acid dissolves practically at once. As soon as a clear solution is obtained, cool under running water, with shaking, and add distilled water to a volume of 500 cc. Add 25 cc. of formaldehyde, and after shaking to insure thorough mixing, acidify by the addition of 3 cc. of glacial acetic acid. Shake to remove most of the carbon dioxide, dilute to volume and mix. Fill up to the neck a series of small bottles, 100 to 150 cc., cork very tightly, label with the date and keep in a cool, dark place. Stored in this manner the stock solution keeps indefinitely.

The working standard is prepared in the following manner:

Transfer with an Ostwald pipette 1 cc. of the stock solution above, containing 1 mg. of uric acid, to a 250-cc. volumetric flask. Half fill the flask with distilled water and add 10 cc. of the 0.66 N sulfuric acid used in the blood precipitation. Mix and add 1 cc. of 40 per cent formaldehyde. Mix, dilute to volume, and mix. This standard contains 0.02 mg. of uric acid per 5 cc. and should be prepared fresh every two weeks.

(b) Sodium Cyanide.- Five per cent solution containing 2 cc. of concentrated ammonia per liter. This should be prepared fresh once a month and when not in use should be kept in the refrigerator.

(c) Arsenic-phosphoric-tungstic Acid Reagent.- Place 100 gm. of pure sodium tungstate in a liter flask and dissolve in about 500 cc. of distilled water. Add 50 gm. of pure arsenic pentoxide ( $As_2O_5$ ), 25 cc. of 85 per cent phosphoric acid, and 20 cc. of concentrated hydrochloric acid. Boil the mixture for twenty minutes, cool and dilute to 1 liter with distilled water. This reagent will keep for one year at room temperature.

2. Procedure.- Place 5 cc. of the protein-free blood filtrate, corresponding to 0.5 cc. of blood, in a test tube, and add 5 cc. of distilled water; place 5 cc. of the uric acid standard containing 0.02 mg. of uric acid in a second test tube, and add 5 cc. of distilled water; then to each tube add 4 cc. of the 5 per cent sodium cyanide solution containing ammonia.

The test tubes need not be graduated, but should be of uniform size and diameter. Before proceeding further, one should make sure that the water bath is boiling, and that a vessel of cold water is also at hand.

Then to the tube containing the standard add 1 cc. of the arsenic-phosphoric-tungstic acid reagent, mix the contents of the tube by inverting once, and quickly immerse in the boiling water. Immediately add 1 cc. of the same reagent to the unknown and after mixing by one inversion, quickly immerse this tube also in the boiling water.

The tubes must be placed in the boiling water immediately after the addition of the arsenic-phosphoric-tungstic acid reagent; delay here is fatal to the determination, as it makes for the development of turbidity. The time elapsing between the immersion of the two tubes must not exceed one minute; a longer interval results in disproportionate color development; and this



latter fact makes it better when there are a number of filtrates to be examined, to limit the number run against one standard preparation to three or, at most, four.

Let the tubes remain in the boiling water for exactly three minutes from the time of immersion of the last tube; longer heating tends to fade the color; then remove, and place in a large beaker of cold water; let stand in the cold water for three minutes, and compare in the colorimeter.

Benedict advises making the color comparisons within five minutes after removing from the cold water, stating that long standing before reading may lead to development of turbidity. Perhaps more important is the immediate immersion of the tubes in the boiling water bath after adding the reagent.

3. Calculation.- Using the weaker standard, as in the above technique, the reading of the standard, usually 20 mm.; multiplied by 4, and divided by the reading of the unknown, gives the uric acid in mg. per 100 cc. of blood.

## VII. Determination of Sugar (Folin and Wu).-

### A. In Blood.

1. Reagents.- (a) Stock Sugar Solution.- One per cent anhydrous dextrose in 0.25 per cent benzoic acid. This solution, when prepared with 0.25 per cent benzoic acid, will not decompose. Two working standards are prepared from this stock standard. The weaker solution contains 0.1 mg. of sugar per cc. and is prepared by diluting 5 cc. of the stock solution to 500 cc. with 0.25 per cent benzoic acid. The stronger standard is prepared by diluting 5 cc. of the stock solution to 250 cc. with 0.25 per cent benzoic acid solution.

(b) Alkaline Copper Solution.- Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 cc. of distilled water in a liter flask. Add 7.5 gm. of tartaric acid, and when the latter has dissolved, add 4.5 gm. of crystallized copper sulfate. Mix and make up to the liter mark with distilled water.

(c) Molybdate-phosphate Solution.- Place 35 gm. of molybdic acid and 5 gm. of sodium tungstate in a liter beaker. Add 200 cc. of 10 per cent sodium hydroxide solution and 200 cc. of distilled water. Boil vigorously for twenty to forty minutes. Cool, dilute to about 350 cc. with distilled water and add 125 cc. of concentrated (85 per cent) phosphoric acid. Dilute to 500 cc. with distilled water.

2. Procedure.- Place 2 cc. of the protein-free blood filtrate, corresponding to 0.2 cc. of blood, in a special blood sugar test tube; these tubes are graduated at 25 cc. and are constricted toward the bottom so as to form a bulb which will contain 4 cc., this amount of fluid rising to just within the constricted portion of the tube. In two other similar tubes place 2 cc. of the standard sugar solutions containing, respectively, 0.2 and 0.4 mg. of dextrose. To each of the three tubes add 2 cc. of the alkaline copper solution.

The surfaces of the mixtures must now have reached the constricted parts of the tubes and must not lie above these parts. Tubes of either too large or too small capacity should be discarded; the surface of the mixture should lie within the constriction.

Transfer the tubes to a boiling water bath, and heat for six minutes; then transfer to a cold water bath and allow to cool, without shaking, for two or three minutes.

Add to each tube 2 cc. of the molybdate-phosphate solution. The cuprous oxide dissolves rather slowly if the amount present is large; but the whole, up to the quantity given by 0.8 mg. of dextrose, dissolves usually within two minutes.

When the cuprous oxide is dissolved, dilute the resulting blue solutions to the 25 cc. mark with a 1:4 dilution of the molybdate-phosphate reagent, and mix each tube thoroughly by inverting several times, using care to insure complete mixing, as the greater part of the color is developed in the bulb of the tube. Compare in the colorimeter, using the standard which more nearly approximates the unknown.

3. Calculation.- When the weaker standard is used, i. e., the one containing 0.2 mg. of dextrose, the reading of the standard, usually 20 mm., multiplied by 100 and divided by the reading of the unknown, gives the sugar in mg. per 100 cc. of blood. When the stronger standard is used, substitute 200 for the 100 in the preceding.

B. In Cerebrospinal Fluid (Lyttle and Hearn).-

1. Reagents.- Solutions required are the same as those used in the Folin-Wu method for the precipitation of blood proteins and determination of blood sugar.

2. Procedure.- Four volumes of spinal fluid are added to 14 volumes of distilled water, and to this mixture 1 volume of 10 per cent sodium tungstate is added, followed by 1 volume of 0.66 N sulfuric acid. Shake and allow to stand for ten minutes; then filter. The sugar determination is carried out on 2 cc. of this filtrate in exactly the same manner as in determining blood sugar.

3. Calculation.- Where the lower standard has been used, the reading of the standard, usually 20 mm., multiplied by 50 and divided by the reading of the unknown equals mg. of sugar per 100 cc. of spinal fluid. When the higher standard is used, substitute 100 for the 50 above.

VIII. Glucose Tolerance Test.

Following the ingestion of a definite amount of glucose, blood sugar is determined at intervals. Urine specimens taken at the same time, are tested for glucose and, if positive, the amount present is determined.

1. Reagents.- Those for blood sugar and urine sugar determination



and in addition

(a) Glucose.- The dosage used at present is 100 gm. regardless of body weight, except in children and in persons differing markedly from normal in stature and general muscular build. It is given in 50 per cent solution to the fasting patient. Lemon juice makes the sugar solution more palatable.

2. Procedure.- Obtain blood and urine specimens on the fasting patient. Give the glucose solution and note the time. Half an hour, one hour, two hours and three hours after the ingestion of the glucose take blood and urine specimens.

Determine the blood sugar on each specimen and test all urines for glucose. Determine the urinary glucose in any positive specimens.

3. Result.- Record the blood and urine glucose for the fasting, half hour, one hour, two hour and three hour specimens, and also the amount of glucose given.

#### IX. Determination of Chlorides (Whitehorn).-

1. Reagents.- (a) Standard Silver Nitrate.-M/35.46 solution. Dissolve 4.791 gm. of C.P. silver nitrate in several hundred cc. of distilled water in a liter volumetric flask. Make up to 1 liter with distilled water, mix thoroughly and preserve in a brown bottle. One cubic centimeter of this solution corresponds to 1 mg. of chlorine.

(b) Standard Potassium or Ammonium Thiocyanate.- M/35.46 solution. Dissolve 3 gm. of potassium thiocyanate (KCNS), or 2.5 gm. of the ammonium salt ( $\text{NH}_4\text{CNS}$ ) in a liter of distilled water. Titrate against 10 cc. of the standard silver nitrate solution using about 0.3 gm. of powdered ferric ammonium sulfate as an indicator; adjust the solution as may be indicated by the titration, so that it will exactly correspond in strength to the silver nitrate solution. The end point in this titration should be the same as that used in the determination of urine chlorides.

(c) Powdered Ferric Ammonium Sulfate ( $\text{FeNH}_4(\text{SO}_4)_2$ ).

(d) Concentrated Nitric Acid.- Specific gravity 1.42; halogen-free.

2. Procedure.- Pipette 10 cc. of the protein-free blood filtrate, corresponding to 1 cc. of blood, into a porcelain dish.

Add 5 cc. of the standard silver nitrate solution and stir thoroughly; add about 5 cc. of the concentrated nitric acid, mix and let stand for five minutes to permit flocking out of the silver chloride. It is to be noted that the silver nitrate and nitric acid are not added to the filtrate simultaneously; to do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction.

Then, with a spatula, add an abundant amount, about 0.3 gm., of the ferric ammonium sulfate, and titrate the excess of silver nitrate with the standard

thiocyanate solution until the definite salmon-red, not yellow, color of the ferric thiocyanate persists, in spite of stirring, for at least ten seconds.

3. Calculation.- Each cc. of the thiocyanate solution corresponds to 1 cc. of the silver nitrate solution; therefore, the amount of thiocyanate used represents the excess of silver nitrate remaining after the reaction with chloride was complete.

Since 5 cc. of silver nitrate were originally used, the difference between 5 and the amount of thiocyanate used for the titration, i. e., 5 minus the titer, gives the amount of silver nitrate solution which was used up in the chloride precipitation. Since this solution was so made that 1 cc. of it corresponds to 1 mg. of chlorine, this figure (5 minus the titer) gives the chlorine in mg. in the filtrate used, representing 1 cc. of blood.

It is preferable to report chlorides as sodium chloride, rather than as chlorine; and the chlorine value above is converted to NaCl by dividing by 0.606. This latter figure, of course, must be multiplied by 100 to give the value per 100 cc. of blood.

The actual calculation can be shortened to the following:

5 minus the titer, in cc., multiplied by 165, will give the chlorides, as NaCl in mg. per 100 cc. of blood.

#### X. Determination of Cholesterol (Leiboff).-

1. Reagents.- (a) Standard Solution of Cholesterol in Chloroform.- A stock solution is first prepared by dissolving 0.160 gm. of pure cholesterol in 100 cc. of redistilled chloroform. The working standard is made by diluting 5 cc. of the stock standard to 100 cc. with chloroform. Five cubic centimeters equal 0.4 mg. of cholesterol. Preserve in dark-glass bottles, preferably in the refrigerator.

(b) Acetic Anhydride.- If not clear and colorless it may be purified by redistillation.

(c) Concentrated Sulfuric Acid.

(d) Asbestos cloth, medium weave.- As purchased this cloth is quite dirty. Cut into strips and extract in a Soxhlet apparatus for an hour or two with chloroform. Dry thoroughly and cut into 2 cm. squares.

2. Procedure.- Place a square of asbestos cloth in the special Leiboff extraction tube. From a pipette drop 0.25 cc. of oxalated blood on to it, slowly. Dry in a vacuum desiccator over calcium chloride for an hour. Introduce 5 cc. of chloroform into the extraction tube, connect to a reflux condenser and immerse in a beaker of water heated over a hot plate and allow to extract for one hour. Detach the tube, remove the cloth, allow to cool to room temperature and add chloroform to the 5 cc. mark. In another tube place 5 cc. of the standard cholesterol solution. To both standard and unknown add 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid.



Mix and place in a beaker of water at a temperature of 20 to 25°C., in a dark cabinet and allow to remain thirty minutes for the color to develop. At the end of this period compare in the colorimeter with the standard set at 15 mm.

3. Calculation.- Twenty-four hundred divided by the reading of the unknown equals mg. of cholesterol per 100 cc. of blood.

Caution.- The color developed by the action of the acetic anhydride and sulfuric acid on cholesterol in chloroform solution changes rapidly on removal from the water bath and exposure to light and since the change in the standard is not at the same rate as the unknown, it is essential that the matching be made as rapidly as possible, within two minutes from the time that the solutions are removed from the dark cabinet. If a series of unknowns are to be read, and this cannot be done in two minutes, it is necessary to prepare another standard.

This reaction is also seriously affected by moisture. It is, therefore, essential that all pipettes, tubes, colorimeter cups and plungers be absolutely dry throughout the procedure.

#### XI. Determination of Inorganic Phosphorus (Fiske and Subbarow).-

1. Reagents.- (a) Sulfuric Acid: 10 N.- Four hundred and fifty cc. of concentrated sulfuric acid added to 1300 cc. of water.

(b) Molybdic Acid Solution.- Dissolve 25 gm. of ammonium molybdate in 200 cc. of water. Rinse into a liter volumetric flask containing 300 cc. of 10 N sulfuric acid. Dilute to the mark with water and mix.

(c) Trichloroacetic Acid: 10 Per Cent. Solution.

(d) Standard Phosphate Solution.- A stock standard is prepared by dissolving 0.3509 gm. of pure monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) in 1 liter of distilled water. Add 10 cc. of chloroform as a preservative and keep in the refrigerator. To prepare the working standard transfer 10 cc. of the stock standard to a 100-cc. volumetric flask, add 80 cc. of trichloroacetic acid and dilute to the mark with distilled water. Five cc. equal 0.04 mg. of phosphorus.

(e) Amino-naphthol-sulfonic Acid Reagent.- Dissolve 30 gm. of sodium bisulfite ( $\text{NaHSO}_3$ ) and 1 gm. of crystalline sodium sulfite ( $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ ) or 0.5 gm. of anhydrous sodium sulfite, in 200 cc. of distilled water. Add 0.5 gm. of purified 1, 2, 4-amino-naphthol-sulfonic acid and stir thoroughly. Preserve in a dark-glass bottle. This reagent should be prepared freshly once a month. The sediment that forms will settle to the bottom and need not be filtered out if care is taken not to stir it up when pipetting.

2. Procedure.- Either serum or plasma is used in the determination, and there must be no hemolysis present. Transfer 8 cc. of trichloroacetic acid to a small Erlenmeyer flask. While the flask is being gently rotated, run in 2 cc. of the serum or plasma from an accurate pipette. Close the mouth of

the flask with a rubber stopper and shake vigorously a few times. Filter through an ashless filter such as Whatman No. 42.

Transfer 5 cc. of the filtrate to a tube graduated at 10 cc. Into a similar tube measure 5 cc. of the standard phosphate solution. To both tubes add 1 cc. of the molybdic acid reagent and 0.4 cc. of the sulfonic acid reagent. Dilute to the mark with distilled water, mix thoroughly, allow to stand ten minutes and compare in the colorimeter.

3. Calculation.- The reading of the standard, usually 20 mm., multiplied by 4 and divided by the reading of the unknown equals mg. of inorganic phosphorus per 100 cc. of blood.

## XII. Determination of Phosphatase (Bodansky Method, Modified).-

1. Reagents.- Those used for phosphorus determination above and in addition

(a) Sodium beta-Glycerophosphate Solution, made by dissolving 1.5 gm. in 50 cc. of water.

(b) Barbitol Solution.- Dissolve 1.030 gm. of sodium barbitol in 50 cc. of water.

These solutions must be prepared the same day they are to be used.

(c) Trichloroacetic acid, 50 per cent solution.

### 2. Procedure.-

(a) Standard.- Place 1 cc. of the stock standard phosphate solution in a small flask or test tube, add 4 cc. of barbitol solution and 2 cc. of distilled water.

(b) Control.- In a similar flask or tube place 2 cc. of clear, un-hemolyzed serum, 4 cc. of barbitol solution and 1 cc. of water.

(c) Unknown.- Similarly place 1 cc. of serum, 2 cc. of barbitol solution and 5 cc. of water in a flask or tube.

Place standard, control and unknown in a beaker or glass filled with water at 37.5°C. to a depth which will cover the mixture in the tubes. Then add to the unknown 1 cc. of the glycerophosphate solution and place the beaker in an incubator at 37.5°C. If available, a water bath set at the same temperature may be used, the glycerophosphate being added to the unknown just as it is placed in the bath. Incubate for exactly two hours.

At the end of this period, add to each tube 1 cc. of the 50 per cent trichloroacetic acid solution. Then to the standard and control, add 2 cc. of the glycerophosphate solution. Filter each mixture through Whatman No. 42 paper, collecting the filtrate in clean tubes.

Source: A. L. Lillie, "The Determination of Phosphatase Activity in Blood"



Carry each filtrate through the phosphorus determination as given in the preceding section, using 5 cc. of each respective filtrate, 1 cc. of molybdic acid solution and 0.4 cc. of the sulfonic acid reagent. Dilute to 10 cc. with water, mix and after standing for ten minutes, compare the unknown and the control against the standard.

3. Calculation.- The reading of the standard, usually 20 mm. multiplied by 4 and divided by the reading of the control gives the mg. of inorganic phosphorus per 100 cc. of serum, (Same as in XI preceding).

The reading of the standard times 8, divided by reading of the unknown gives the mg. of inorganic phosphorus originally present plus that converted from the organic state by the action of the enzyme per 100 cc. of mixture.

The difference between the values for unknown and control, gives the phosphatase units, one unit being that amount which will liberate 1 mg. of inorganic phosphorus per 100 cc. of serum from the organic state in two hours at 37.5°C. Since normal values differ according to the method used, it is essential in reporting to record the normal values of the method used. Upper limits of normal according to the method given here are: For adults, 10 units; for children, 20 units.

### XIII. Determination of Calcium (Roe and Kahn).--

1. Reagents.- In addition to the solutions required for phosphorus determinations, the following are required:

(a) Standard Calcium Solution.- Prepare a stock solution by dissolving 0.4991 gm. of pure calcium carbonate in about 50 cc. of 10 per cent trichloroacetic acid in a 1000-cc. volumetric flask. Iceland spar is preferred, if available. Shake well, and when evolution of  $\text{CO}_2$  has ceased, dilute to the mark with 10 per cent trichloroacetic acid. To prepare the working standard transfer 10 cc. of the stock solution to a 100-cc. volumetric flask, add 70 cc. of 10 per cent trichloroacetic acid and dilute to the mark with distilled water. Five cc. equal 0.1 mg. of calcium.

(b) Alkaline Alcohol Wash Reagent.- In a 100-cc. cylinder place 58 cc. of 95 per cent ethyl alcohol, add 10 cc. of amyl alcohol and make up to 100 cc. with distilled water. Add 2 drops of 1 per cent phenolphthalein and then 5 per cent sodium hydroxide, a drop at a time, with repeated shaking until a distinct pink is obtained.

(c) Sodium Hydroxide: 25 Per Cent Solution.

(d) Trisodium Phosphate,  $\text{Na}_3\text{PO}_4$ : 5 Per Cent Solution.

2. Procedure.- Two cc. of serum are precipitated with 8 cc. of 10 per cent trichloroacetic acid in the same manner as in the phosphorus determination.

Where phosphorus and calcium are to be determined on the same specimen, sufficient filtrate for both determinations may be obtained by precipitating

3 cc. of serum with 12 cc. of the trichloroacetic acid.

To a graduated centrifuge tube, transfer 5 cc. of the filtrate and to another similar tube transfer 5 cc. of the standard calcium solution. The tips of the centrifuge tubes used must be sufficiently narrow so that the diameter at the 0.1 cc. mark will not exceed 7 mm. but must not be too finely drawn out. They must be absolutely clean and when not in use should be kept immersed in the dichromate sulfuric acid cleaning solution. No reliance should be placed on the graduation marks, as they have been found to be inaccurate in many tubes and the 10 cc. mark should be checked. If found inaccurate, a new mark should be made. To both tubes add 1 cc. of 25 per cent NaOH, mix by twirling and allow to stand for five minutes. Then add 1 cc. of the trisodium phosphate solution and allow to stand for one hour to complete precipitation of the calcium phosphate.

Centrifugalize for two minutes. Decant the supernatant fluid with one smooth movement that will not disturb the precipitate. With the mouth still inverted, the tube is placed upon a clean filter paper and allowed to drain several minutes, after which time any remaining fluid which may have collected on standing is removed from within the mouth of the tube by touching it with a slip of filter paper or by a clean piece of gauze. Add from a pipette about 3 cc. of alkaline alcohol wash reagent in such a manner as to break up the mat of  $\text{Ca}_3(\text{PO}_4)_2$  in the bottom of the tube. This is done by using a bulb pipette with a fine tip and blowing forcefully, directing the stream upon the calcium phosphate precipitate. If the calcium phosphate mat is not broken up completely by this procedure, it must be fragmented thoroughly with a clean glass stirring rod. The walls of the tube are now washed down with an additional 2 cc. of the alkaline alcohol wash reagent. The tubes are centrifugalized again for two minutes, then decanted and drained as above.

Redissolve the precipitate in both standard and unknown in 4 cc. of 10 per cent trichloroacetic acid, add to each tube 1 cc. of the molybdic acid reagent, the same as is used in the phosphorus determination, and 0.4 cc. of the sulfonic acid reagent, also the same as for the phosphorus determination. Dilute to the 10 cc. mark with distilled water, mix, allow to stand ten minutes, then compare in the colorimeter.

3. Calculation.- The reading of the standard multiplied by 10 and divided by the reading of the unknown equals the mg. of calcium per 100 cc. of blood.

Caution.- This reaction is a reaction for phosphorus based upon the amount of phosphorus contained in the calcium phosphate precipitate. It has been found impossible to obtain reagents which are absolutely calcium-free or free of other substances which would also give color. Therefore, a standard calcium solution is prepared and treated in the same manner throughout as the blood filtrate. Consequently, any error produced in the blood calcium determination as a result of color-producing substances in the reagents used will be exactly balanced by a similar error in the standard used, provided the standard is treated in the same manner as the unknown and with the same reagents. Most filter paper contains traces of calcium. It is, therefore, necessary to use calcium-free filter paper in filtering the proteins from the



blood. A double acid-washed paper, such as Whatman No. 42, has been found to meet these requirements. As a result of the action of the 25 per cent NaOH and 5 per cent trisodium phosphate on the glass bottles, a precipitate of silicates will form which will give a color. If kept in tall bottles, the precipitate will settle to the bottom, and the clear supernatant fluid can then be pipetted from the top, otherwise, it is necessary to filter both solutions just before use.

In previous methods calcium was determined by precipitation of the oxalate from the diluted blood serum without prior removal of the blood serum proteins. It has been found that when serum is so precipitated, about 5 to 15 per cent of the calcium fails to be precipitated, consequently when the precipitation is carried out on the protein-free trichloroacetic filtrate, the results will average about 10 per cent higher than those usually published as normal based upon the usual precipitation from the blood serum in the presence of the proteins. The method herein described gives results similar to those obtained with the oxalate precipitation on the protein-free trichloroacetic filtrate. The normals are consequently higher than usually published, being 10 to 12 mg. per 100 cc. of blood instead of 9 to 11 mg.

Should it be necessary to send specimens for calcium and phosphorus determination to a distant laboratory, clear serum may be sent for calcium, but since the action of the enzyme phosphatase continues in the serum, changing organic to inorganic phosphorus, only the trichloroacetic acid filtrate is suitable for this purpose. Since both Ca and P are usually determined together, the filtrate is sent in for both.

#### XIV. Determination of Bile Pigment in Serum.-

##### A. Icterus Index (Bernheim).

##### 1. Reagents.- (a) Potassium dichromate, 1:10,000 solution.

Dissolve 0.1 gm. of chemically pure potassium dichromate in about 500 cc. of distilled water in a liter volumetric flask. Add 4 drops of concentrated sulfuric acid and dilute to the mark with distilled water. Keep in a dark glass bottle in the dark. This is the standard against which the color of the serum is matched.

##### (b) Sodium chloride, 0.9 per cent solution.

2. Procedure.- Accurately dilute 1 cc. of clear serum with the sodium chloride solution until its color matches the standard approximately. This dilution may be 1:2, 1:5, 1:10 or even more. Place the standard in one colorimeter cup and set it at 15 mm. Place the serum, undiluted or diluted as necessary, in the other cup and match against the standard.

3. Calculation.- The reading of the standard, 15 mm., divided by the reading of the serum, times the dilution, if any, gives the icterus index.

Note.- Since this test is simply a measurement of the color of the serum, it is evident that even the slightest trace of hemolysis will vitiate the

add 2 cc. of concentrated hydrochloric acid, mix and let stand several minutes. Centrifugalize (or filter) and pour the clear supernatant into a colorimeter cup and compare with the bilirubin standard.

3. Calculation.- The reading of the standard, times 10, divided by the reading of the unknown equal mg. bilirubin per 100 cc. of serum or plasma. One unit of bilirubin is 1 part in 200,000 or 0.5 mg. per 100 cc.

Note.- The normal icterus index is from 4 to 6. Latent jaundice (hyperbilirubinemia without apparent jaundice) gives readings between 6 and 15. With an icterus index of 15 and over, visible jaundice occurs.

By the quantitative test, normal serum contains from 0.2 to 0.5 units of bilirubin (0.1 to 0.25 mg. per 100 cc.) and the serum of cases of latent jaundice contains from 0.5 to 2 units (0.25 to 1.0 mg. of bilirubin per 100 cc.).

XV. Determination of Blood Proteins (Andersch and Gibson Modification of the Method of Wu and Ling).

#### A. Determination of Serum Proteins.

Ordinarily it is the serum proteins which are determined, that is, albumin and globulin. The method given below determines total protein and albumin directly, the globulin being found by difference.

When it becomes necessary to determine fibrinogen, oxalated plasma must be used. The fibrinogen is precipitated as fibrin and separated from the plasma solution. It is then determined directly as given under B, Determination of Fibrinogen in Plasma.

##### 1. Reagents.-

- (a) Trichloroacetic Acid: 20 per cent solution.
- (b) Sodium Hydroxide: 10 per cent.
- (c) Sodium Chloride: 0.9 per cent.
- (d) Standard Tyrosine Solution. Dissolve 200 mg. of pure dry tyrosine in 1 liter of 0.1 N HCl.
- (e) Saturated Sodium Carbonate Solution.
- (f) Saturated Ammonium Sulfate Solution. Fifty-six gm. per 100 cc.
- (g) Phenol Reagent of Folin and Ciocalteu.

Transfer 100 gm. of sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) and 25 gm. of sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ) together with 700 cc. of water to a 1500-cc. Florence flask. Add 50 cc. of 85 per cent phosphoric acid and 100 cc. of concentrated hydrochloric acid. Connect with a reflux condenser by means of a cork or rubber stopper wrapped in tinfoil, and boil gently for ten hours. At the end of the boiling period add 150 gm. of lithium sulfate, 50 cc. of





water and a few drops of bromine. Boil the mixture without the condenser for about fifteen minutes to remove the excess bromine. Cool, dilute to 1 liter and filter. The finished reagent should have no greenish tint, as this means the presence of blue reduction products which will lessen the range of true proportionality.

2. Procedure for Total Serum Proteins.- The serum is diluted 1 to 10 with 0.9 per cent NaCl. One cubic centimeter of this dilution is transferred to a 15-cc. centrifuge tube and 4 cc. of water added, followed by 1 cc. of the 20 per cent trichloroacetic acid. The precipitate is centrifugalized out and the supernatant fluid discarded. The precipitate is dissolved in 0.5 cc. of 10 per cent NaOH and heated in a boiling water bath for thirty minutes. Two cubic centimeters of the standard tyrosine solution are measured into a centrifuge tube similar to the one containing the unknown. Both tubes should be accurately graduated at the 10 cc. mark. Dilute both standard and unknown to the 5 cc. mark with distilled water, add 1 cc. of the phenol reagent and 3 cc. of saturated sodium carbonate solution. Make up both to the 10 cc. mark with distilled water, mix and compare in the colorimeter after standing for thirty minutes.

3. Procedure for Albumin.- To 1 cc. of serum add 4 cc. of water and 5 cc. of saturated ammonium sulfate solution and place in an incubator at 37°C. for fifteen minutes, then filter through a fine filter paper such as Whatman No. 42; if the filtrate is not clear, return to the paper.

To 2 cc. of the filtrate in a centrifuge tube accurately graduated at 10 cc., add 3 cc. of water and 1 cc. of the 20 per cent trichloroacetic acid. Centrifugalize, pour off the supernatant fluid, dissolve the precipitate in 0.5 cc. of 10 per cent NaOH; heat in the boiling water bath for thirty minutes, then develop the color and compare against a standard in the same manner as described above for total protein.

4. Procedure for Globulin.- The per cent of globulin is obtained by subtracting the albumin from the total protein.

5. Calculations.- (a) For Total Protein.- The reading of the standard, usually 10 mm., multiplied by 5.2 and divided by the reading of the unknown equals the per cent of total protein.

(b) For Albumin.- The reading of the standard multiplied by 2.58 and divided by the reading of the unknown equals the per cent of albumin.

(c) For Globulin.- Per cent of total protein minus per cent of albumin equals per cent of globulin.

#### B. Determination of Fibrinogen.

1. Reagents.- The same as in A.

2. Procedure.- To 1 cc. of plasma from oxalated blood in a 15-cc. centrifuge tube, add 2 cc. of water and 1 cc. of saturated ammonium sulfate



solution. Mix, let stand for a few minutes, then centrifugalize to throw down the precipitated fibrinogen (fibrin). Pour off the supernatant fluid completely. Dissolve the fibrin precipitate in 0.5 cc. of 10 per cent sodium hydroxide and heat in the boiling water bath for thirty minutes. For the standard, place 2 cc. of the tyrosine solution in another centrifuge tube. Dilute both standard and unknown to the 5 cc. mark, add to each 1 cc. of the phenol reagent, and then 3 cc. of saturated sodium carbonate solution. Make both up to the 10 cc. mark with water, mix and let stand for thirty minutes. Compare in the colorimeter, with the standard set at 10 mm.

3. Calculation.- The reading of the standard, times 0.52, divided by the reading of the unknown equals per cent of fibrinogen.

Note.- When fibrinogen, albumin and globulin are desired on the same case, secure enough oxalated blood to give at least 2 cc. of clear plasma. Determine fibrinogen as above on a 1-cc. portion. Treat another 1-cc. portion exactly as serum is treated in A. In this case the total protein equals albumin plus globulin plus fibrinogen. Having the figures for total protein, albumin and fibrinogen, the globulin is found by difference.

XVI. Note.- Alkali Reserve.- See Laboratory Methods, U. S. Army, 4th Edition, 1935.

#### XVII. Determination of Carbon Monoxide.-

A. Carbon Monoxide in Blood (Sayers, Yant and Jones).- This method depends upon the fact that carbon monoxide, CO, combines with the hemoglobin of the blood, displacing the oxygen and changing oxyhemoglobin, HbO, to carbon monoxide hemoglobin, HbCO. The blood under examination is treated with absolutely fresh pyrotannic acid solution, which in the presence of CO develops a specific color. This color is compared with a set of standards representing various degrees of saturation with CO from 0 per cent to 100 per cent in steps of 10.

1. Reagents.- (a) Pyrogallol: 2 per cent aqueous solution.

(b) Tannic Acid: 2 per cent aqueous solution. Equal volumes of (a) and (b) are mixed just prior to use.

(c) Preparation of Standards.- Five cubic centimeters or more of human or animal blood are drawn and kept from clotting by the addition of 50 mg. of potassium citrate per 10 cc. of blood. This blood is divided into equal parts, one of which is immediately diluted 1 to 10 with distilled water, forming Solution I, which is all oxyhemoglobin; the other is saturated with CO by bubbling ordinary illuminating gas through it. Where illuminating gas is not available, CO may be generated by heating sulfuric acid with oxalic acid, passing the gas produced through a solution of sodium hydroxide to remove the carbon dioxide, before passing it through the blood. The saturated blood is now diluted 1 to 10 with distilled water, forming Solution II, all carbon monoxide hemoglobin.

From Solution I and Solution II, mixtures are made in small test tubes,

approximately 8 mm. in diameter; which total 1 cc. but vary from 0 per cent to 100 per cent HbCO in steps of 10. For example: To the first tube 1 cc. of the oxyhemoglobin, Solution I only, is added; to the second tube 0.9 cc. of Solution I and 0.1 cc. of Solution II; to the third tube 0.8 cc. of Solution I and 0.2 cc. of Solution II and so on in each of the succeeding tubes, Solution I diminishing by 0.1 cc. in each tube, and Solution II increasing by 0.1 cc. in each tube until the last tube which contains 1 cc. of Solution II only and will represent 100 per cent HbCO. Thus, the percentage of HbCO increases from 0 per cent in the first tube, by steps of 10 per cent in each succeeding tube, to 100 per cent in the last tube.

To each standard thus prepared add 1 cc. of the mixture of equal parts of strictly fresh solutions of 2 per cent pyrogalllic acid and 2 per cent tannic acid, after which the tube is inverted twice to insure thorough mixing. The tube should be sealed immediately by pouring a little melted paraffin on top of the contents while immersed in cold water to prevent overheating. When the walls of the tube become dry the remainder of the tube should be filled with ordinary sealing wax. Care should be taken to exclude all air. These standards develop their full color in about thirty minutes, and if kept in a cool dark place, will keep for two weeks or more.

2. Procedure.- Collect 0.1 cc. of blood from the tip of the finger and dilute with 0.9 cc. of distilled water in a test tube of the same size used in the preparation of the standards. Add 1 cc. of the strictly fresh pyrotannic acid mixture used in the preparation of the standards. This mixture must be made fresh every day. Invert the tube several times. Place in a rack and allow to stand for fifteen minutes, at the end of which period comparison is made with the standards by interposing between them until the standard is found which most nearly matches. If CO is indicated the tube should be allowed to stand for fifteen minutes longer and another reading made.

3. Calculation.- The percentage of HbCO is estimated from the value of the standard most closely matched.

#### B. Carbon Monoxide in Air (Sayers, Yant and Jones).-

1. Reagents.- Same as in A, Carbon Monoxide in Blood.

2. Procedure.- Fresh human or animal blood is obtained and diluted 1 to 10 with distilled water. Two cubic centimeters of this solution are then introduced into a sample bottle containing the air to be analyzed, the manipulation being such as to allow as little of the air as possible to escape.

The same bottle is a 250-cc. bottle or flask in which the air to be analyzed is collected by inserting the glass tube on the end of a scrubber containing soda lime and pumping air through the scrubber and bottle by means of an aspirating bulb. At the last squeeze of the bulb, the glass tube is quickly removed and a rubber stopper inserted in the bottle.

The bottle containing the blood is equilibrated by being rotated constantly for fifteen to twenty minutes, as much as possible of the surface of



the bottle being covered with the blood solution. When this equilibration has been finished, 1 cc. of the equilibrated blood solution is placed in a test tube, 1 cc. of fresh pyrotannic acid mixture added, and the comparison of colors and determination of HbCO made according to the procedure for blood.

3. Calculation.-  $\frac{\text{HbCO}}{100-\text{HbCO}} \times \frac{2093}{300} = \text{parts of CO per 10,000 parts of air.}$

Example.- A blood solution equilibrated with a gas sample was found to contain 50 per cent HbCO by comparison with the standards. Substituting this value in the above equation gives the following:  $\frac{50}{100-50} \times \frac{2093}{300} = 7 \text{ parts CO per 10,000 parts of air.}$

The equilibration of the blood with the air sample should be done at a temperature not varying more than 3°C. plus or minus from 20°C. (5° plus or minus from 68°F.). Within this range the temperature correction is so small as to be negligible.

#### XVIII. Determination of Ethyl Alcohol in Blood, Urine and Spinal Fluid.-

The method used is a modification of that originally devised by the French toxicologist, Nicloux. It consists of the oxidation of the ethyl alcohol by means of potassium dichromate and sulfuric acid with the coincident reduction of the dichromate to chromium sulfate to a degree corresponding to the amount of alcohol oxidized. The chromium salt is green in color, and with small quantities of alcohol the intensity of the green color measures exactly the amount of alcohol oxidized.

Various methods for preparing the standards have been used in the past, all of them having certain objectionable features. A combination of methods has been worked out obviating some of the earlier difficulties, using a stronger reagent than that given in various laboratory manuals and journals. For that reason, the Anstie's reagent as given here, called "Anstie's reagent, modified, stronger" must not be confused with that used in other methods. This newer reagent is about 11 per cent stronger than the older modified reagent. The new reagent may be converted into the older one by diluting 9 parts of the new reagent with 1 part of water.

1. Reagents.- (a) Anstie's Reagent, modified, stronger, is made as follows: Dissolve 3.70 gm. of chemically pure, reagent quality potassium dichromate in 150 cc. of distilled water. Add slowly, with constant stirring, 280 cc. of concentrated sulfuric acid, chemically pure. Dilute to 500 cc. with distilled water.

(b) Standard Alcohol Solution.- Place about 50 cc. of distilled water in a 100 cc. volumetric flask. To it add 2.53 cc. of absolute ethyl alcohol by means of an accurately graduated pipette, keeping the pipette tip near the surface of the water to prevent loss of alcohol by evaporation. Make up to 100 cc. with distilled water.

(c) Scott-Wilson Reagent.-

(1) Mercuric cyanide, 5 gm. dissolved in 300 cc. of water.

(2) Sodium hydroxide, 90 gm. dissolved in 300 cc. of water.

(3) Silver nitrate, 1.45 gm. dissolved in 200 cc. of water.

Add (2), thoroughly cooled to (1); mix thoroughly, then add (3) to the mixture with constant stirring. This solution will keep for six months. If it becomes cloudy, or a precipitate forms, filter it. Never pipette this solution - it is very poisonous.

(d) Color Comparison Standards.

Arrange 9 test tubes of uniform diameter and color (color comparison tubes are better) in a test tube rack and place 9 cc. of Anstie's reagent in each tube. Then add to each tube the standard alcohol solution and distilled water in the amounts shown in the following table:

<u>Tube No.</u>	<u>Alcohol Solution</u>	<u>Distilled Water</u>	<u>Corresponds to Alcohol in the Specimen</u>
1	None	1.0 cc.	Negative
2	0.1 cc.	0.9 cc.	0.5 milligrams per cubic centimeter
3	0.2 cc.	0.8 cc.	1.0 " " " "
4	0.3 cc.	0.7 cc.	1.5 " " " "
5	0.4 cc.	0.6 cc.	2.0 " " " "
6	0.5 cc.	0.5 cc.	2.5 " " " "
7	0.6 cc.	0.4 cc.	3.0 " " " "
8	0.7 cc.	0.3 cc.	3.5 " " " "
9	0.8 cc.	0.2 cc.	4.0 " " " "

The contents of each tube must then be thoroughly mixed. This may be accomplished by drawing the contents of each tube up into a 10 cc. pipette and allowing it to run back into the tube several times.

These standards may be kept for several weeks if tightly stoppered and kept in a vertical position in a test tube rack. The solutions in the standards must not come into contact with the stoppers, as both cork and rubber stoppers contain reducing substances which may cause a change in color of the standards.

Each standard should be labeled with the number of milligrams of alcohol to which it corresponds (e.g., tube No. 1 should be labeled "Negative"; tube No. 5 should be labeled "2.0 mg."; etc.).

It is useless to try to make standards for readings greater than 4.0 milligrams of alcohol per cubic centimeter, as the Anstie's reagent is apparently completely changed by that amount of alcohol, and no difference can be detected between the 4.0, the 4.5 and 5.0 mg. per cc. standards no matter by which method they are prepared. Should specimens be encountered which have 4.0 mg. of alcohol per cc., or more, a second determination should be made using half the quantity of the specimen (2 cc. instead of 4 cc.) and the result multiplied by two to give the final reading.



## 2. Procedure.- Urine, blood or spinal fluid.

Arrange two 25 x 210 mm. tubes with two-holed rubber stoppers and inlet and outlet tubes. The inlet tubes should extend nearly to the bottom of the tube and the outlet tube just below the stopper. Using well-washed rubber tubing, connect the inlets and outlets in such a manner that a current of air may be aspirated through the specimen tube over into the tube containing Anstie's reagent.

In the specimen tube place 4 cc. of specimen, 2 to 4 cc. of Scott-Wilson reagent, and sufficient water to make 10 cc. Half way between the upper level of the fluid contents and the bottom of the stopper, place a wad of glass wool.

In the second tube place 10 cc. of the Anstie's reagent.

Stopper the tubes, and adjust the suction so that a reasonable current of air is aspirated through the tubes. Immerse the tubes in a water bath previously brought to boiling. Continue the boiling and aspiration for 12 to 15 minutes.

Cool the dichromate solution and compare with the standards which read directly in milligrams per cc.

3. Result.- Report in mg. per cc. as read from the standard matched, each mg. per cc. corresponding to tenths of per cent.

## XIX. Determination of Sulfonamides in Blood and Urine. (Method of Marshall and Litchfield, modified.)

The methods for sulfanilamide, sulfapyridine, sulfathiazole and sulfaguanidine are the same except for the preparation of standards and the calculations. The procedure is based on the diazotization of the drug and subsequent coupling to form a colored compound which is compared colorimetrically with a standard treated in the same manner.

### A. Determination of Sulfanilamide in Blood and Urine.

1. Reagents.- (a) Sodium Nitrite, 0.1 per cent aqueous solution, freshly prepared each day.

(b) Sodium Phosphate, Monobasic, and Ammonium Sulfamate Solution. Dissolve 13.8 gm. of monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) and 0.5 gm. of ammonium sulfamate in water and make up to 100 cc. This solution is one molar in sodium phosphate.

(c) Dimethyl-a-naphthylamine, 0.4 per cent solution in 95 per cent ethyl alcohol. Kept in a dark bottle in the refrigerator, this reagent will keep at least six months.

(d) Trichloroacetic Acid, 15 per cent aqueous solution.

(e) Saponin, 0.05 per cent aqueous solution.

(f) Hydrochloric Acid, 2 N.

(g) Stock Sulfanilamide Solution. Dissolve 100 mg. of pure sulfanilamide crystals in water and make up to 1 liter. Do not use tablets.

(h) Working Standard. Dilute 10 cc. of stock solution to 100 cc. with water. Each cc. of the working standard contains 0.01 mg. sulfanilamide.

## 2. Procedure for Blood.

Take one volume of oxalated blood with 15 volumes of water. Let stand fifteen minutes. If the saponin solution is substituted for the distilled water, laking will be complete in one to two minutes. To the laked blood add 4 volumes of trichloroacetic acid and after standing five minutes, filter.

(a) Free Sulfanilamide. To 10 cc. of filtrate add 1 cc. of the sodium nitrite solution and mix. After three minutes, add 1 cc. of the sodium phosphate-ammonium sulfamate solution, mix and let stand two minutes. Add 5 cc. of the dimethyl-a-naphthylamine solution and after standing ten minutes compare in the colorimeter with a standard prepared at the same time and treated in the same manner. For blood, two standards will cover the range of concentrations ordinarily encountered. The weaker standard consists of 2 cc. of the working standard solution (total 0.02 mg. sulfanilamide), 2 cc. of trichloroacetic acid solution and 6 cc. of water. In the stronger standard use 5 cc. of working standard (0.05 mg. sulfanilamide), 2 cc. of trichloroacetic acid and 3 cc. of water.

(b) Total Sulfanilamide. Treat 10 cc. of blood filtrate prepared as above with 1 cc. of 2 N hydrochloric acid in a boiling water bath for one hour. Cool and adjust the volume to 10 cc. Proceed as for free sulfanilamide except that a 2 molar sodium phosphate - 0.5 per cent ammonium sulfamate solution (27.6 gm.  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  to 100 cc.) is used.

(c) Acetylsulfanilamide. The difference between the total and the free gives the amount of conjugated acetylsulfanilamide.

## 3. Procedure for Urine.

Urine is diluted so as to contain 0.5 to 2.0 mg. per cent of sulfanilamide. A dilution of 1:100 usually is satisfactory. Treat 10 cc. of diluted urine with 1 cc. of the trichloroacetic solution and then proceed in exactly the same manner as for blood. Two standards cover the usual range of concentration in the urine. The weaker consists of 5 cc. of the working standard, 1 cc. of trichloroacetic acid and 5 cc. of water (sulfanilamide content - 0.05 mg.). The stronger standard contains 10 cc. of working standard and 1 cc. of trichloroacetic acid (0.1 mg. sulfanilamide).

## 4. Calculations.- In general the following formula applies:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{Mg. Sulfanilamide in standard used} \times \frac{\text{Dilution of Unknown}}{\text{cc. of filtrate or dilution taken}} \times 100 = \text{mg. per 100 cc.}$$



With the procedure given above, the formula may be reduced to:

For Blood  $\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4$  (if weaker standard is used, = ng. per 100 cc.  
10 if stronger is used)

For Urine  $\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 50$  (if weaker standard is used, = ng. per 100 cc.  
100 if stronger is used)

#### B. Determination of Sulfapyridine in Blood and Urine.

1. Reagents.- Exactly the same as for sulfanilamide.
2. Procedures for Blood and Urine.- Exactly as for sulfanilamide and using the same sulfanilamide working standard.
3. Calculations.- Since sulfapyridine has a lower color-producing value than the sulfanilamide in the standard used (0.8 compared with 1.0) the results must be multiplied by the factor 1.25 to give the true values.

#### C. Determination of Sulfathiazole in Blood and Urine.

The reagents, procedures and calculations are exactly the same as those used in the sulfanilamide determinations except for the standard.

Since the color developed by sulfathiazole differs considerably in shade from that of sulfanilamide, the stock standard must be made by dissolving 100 mg. of pure sulfathiazole crystals in water and making it up to 1 liter.

#### D. Determination of Sulfaguanidine in Blood and Urine.

The reagents, procedures and calculations are exactly the same as for sulfanilamide except for the standard used. The stock standard should be prepared from pure sulfaguanidine crystals.

108 CHLORIDES (Method of Schales and Schales, as modified by Division of Chemistry and Physics, Army Medical School.)

a. REAGENTS:

(1) Standard sodium chloride solution. C.P. sodium chloride is dried at  $120^{\circ}\text{C}$  and 500 mg are weighed out and dissolved in several hundred cc of distilled water in a liter volumetric flask. The solution is made up to 1 liter with distilled water.

(2) Mercuric nitrate solution. 1.5 gm of C.P. mercuric nitrate are dissolved in a few hundred cc of distilled water with the addition of 20 cc of 2N nitric acid and made up to 1 liter volume with distilled water. This solution is titrated against 5 cc of the standard sodium chloride solution, using 4 drops of the indicator, and adjusted as may be indicated by the titration so that it will exactly correspond in strength to the sodium chloride solution.

(3) Indicator. 100 mg of s-diphenylcarbazon are dissolved in 100 cc of 95% ethyl alcohol and stored in a dark bottle, in the refrigerator. A fresh solution should be prepared each month as it is not very stable.

b. PROCEDURE:

(1) Pipette 5 cc of the protein free blood filtrate, corresponding to 0.5 cc of blood, into a porcelain dish. Add 0.06 cc (4 drops) of indicator solution, and titrate with the mercuric nitrate solution, using a microburette calibrated in 0.01 cc intervals. The clear and colorless solution turns an intense violet-blue on the addition of the first drop of mercuric nitrate solution in excess. This color does not fade.

c. CALCULATION:

(1) The reading of the burette multiplied by 100 gives directly the chlorides as sodium chloride in 100 cc of blood.

REFERENCE: Schales, Otto, and Schales, Selma S., Journal of Biol. Chemistry, 140, 879 (1941)

NOTE: "Baker's Analyzed" mercuric nitrate, C.P., is a satisfactory grade. s-Diphenylcarbazon may be secured from the Eastman Kodak Co., Cat. No. 1459. These two reagents may be secured by local purchase or on non-standard requisition.





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[illegible][illegible][illegible]

.....

A series of 12 small, faint line drawings of various insects, including beetles, flies, and bees, arranged in a grid-like pattern. The drawings are simple and appear to be from a scientific or educational text.

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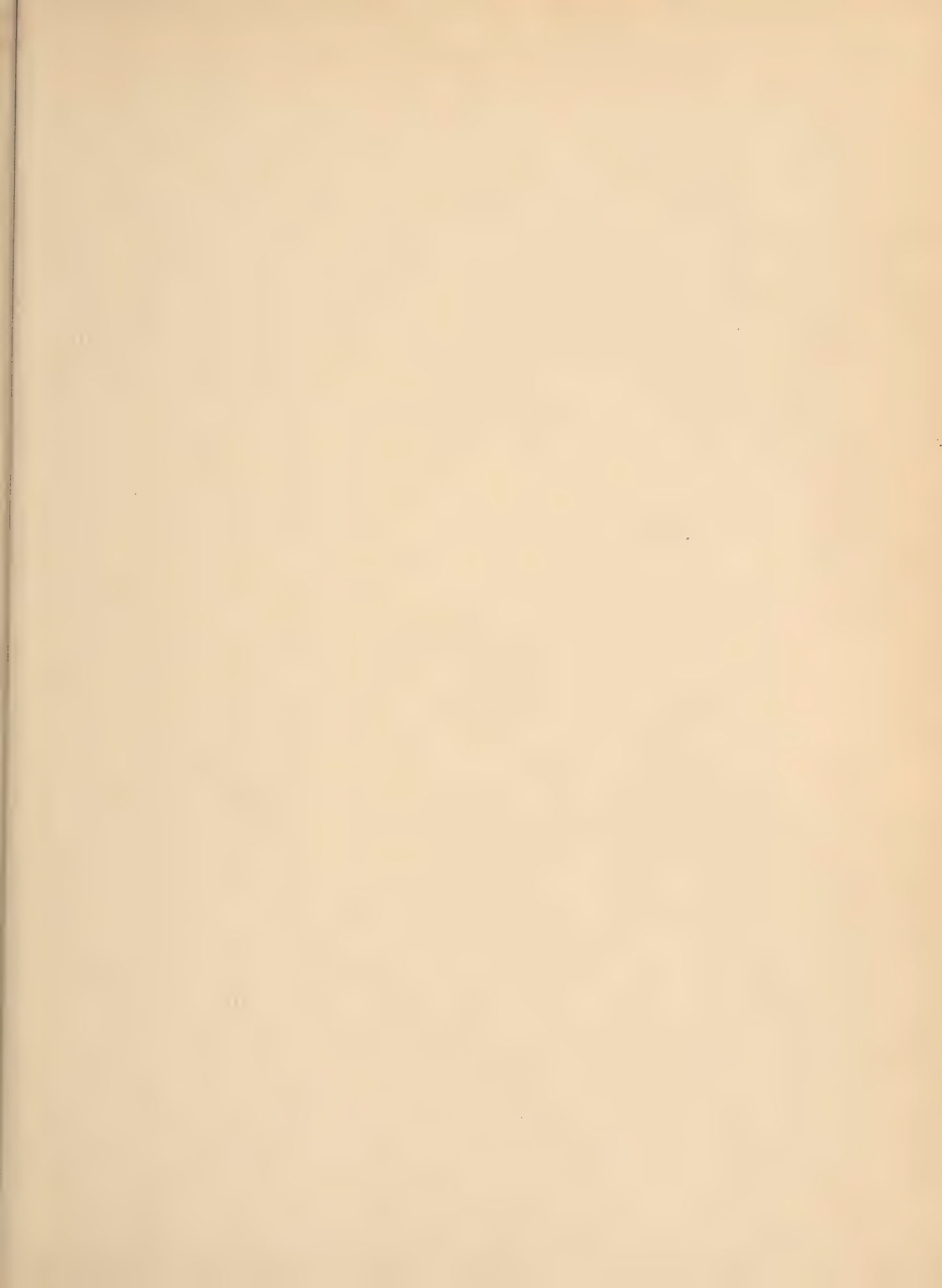
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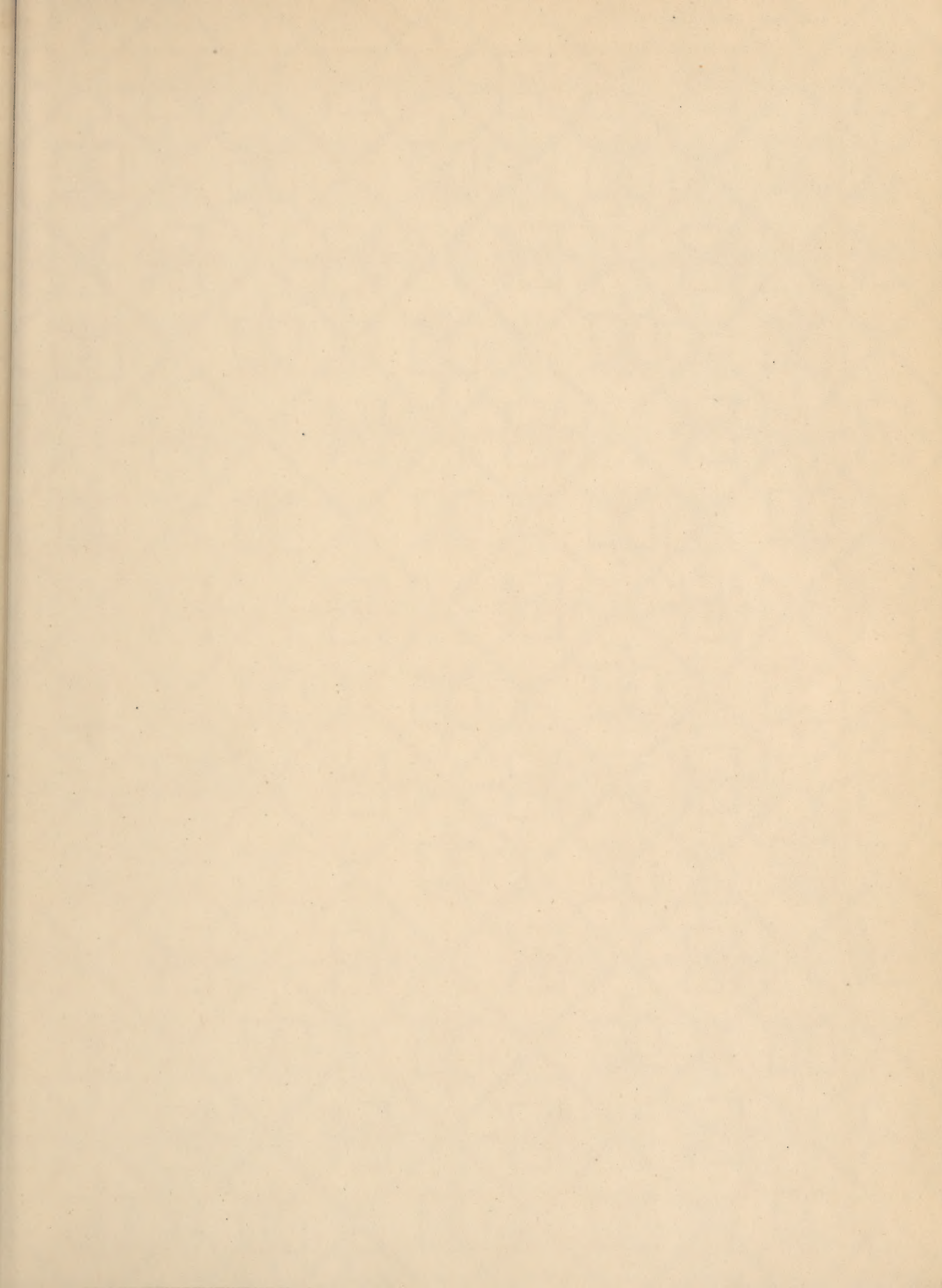
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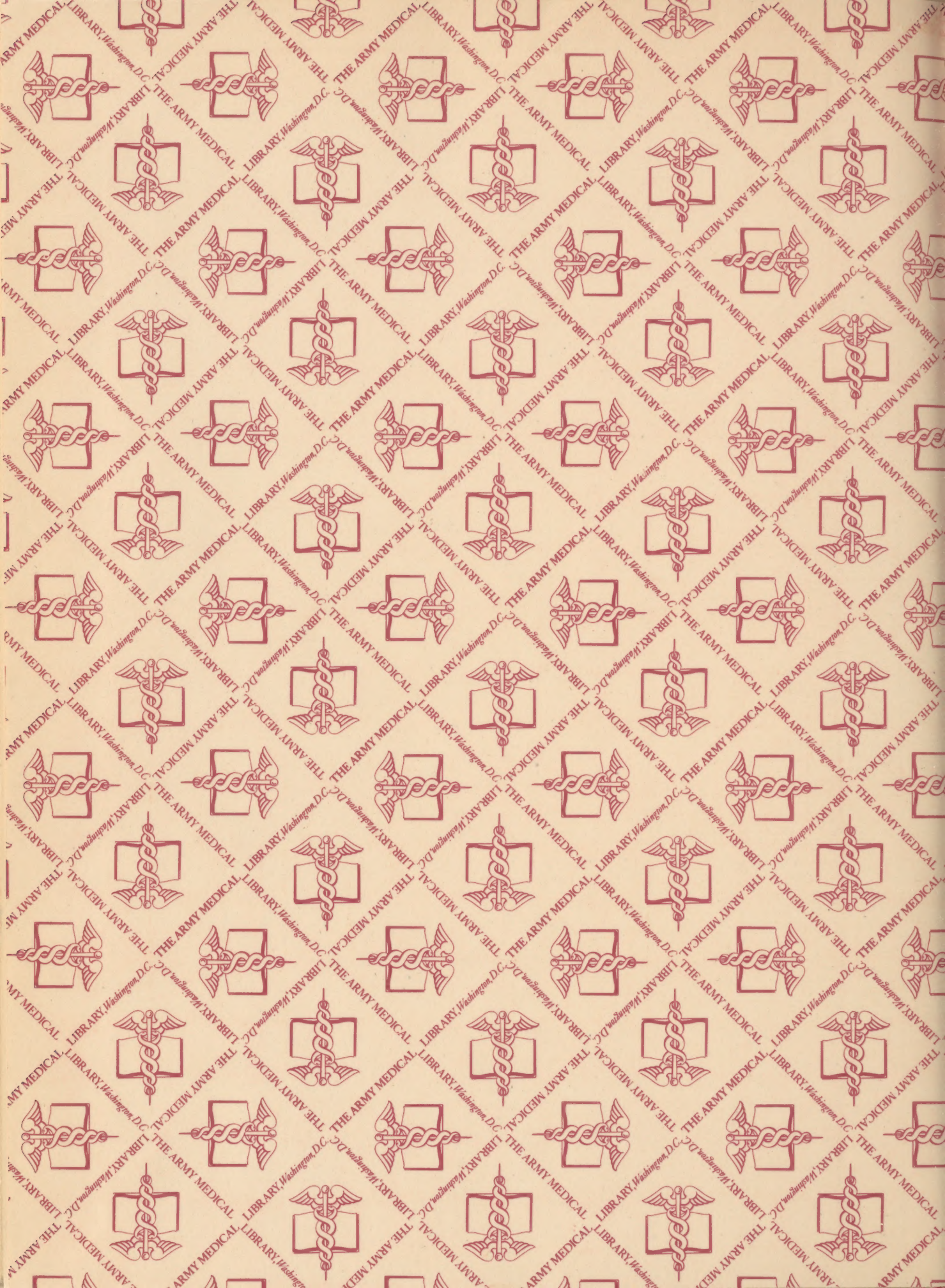




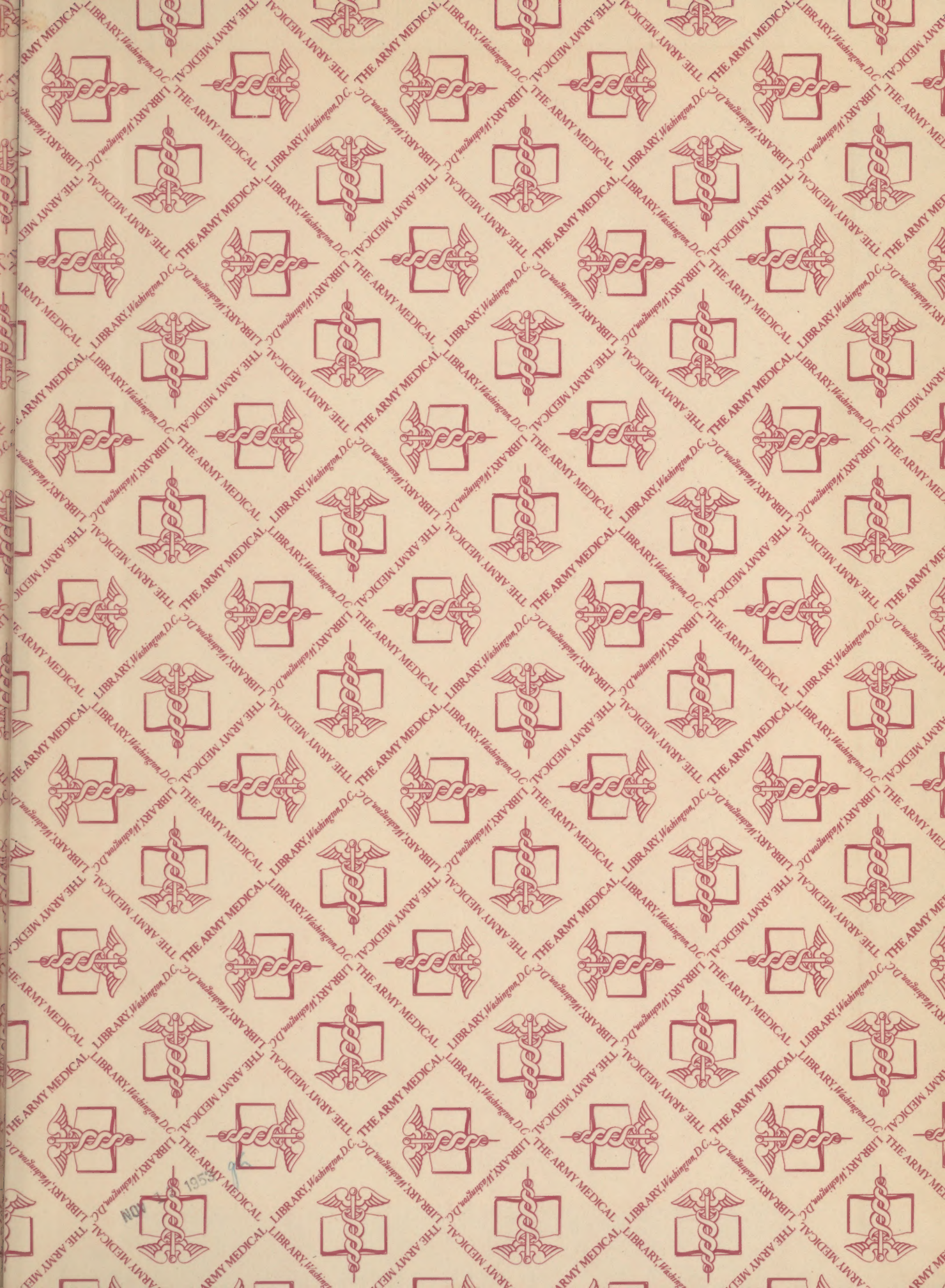














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